UNIVERSITY OF LONDON THESIS

Degree PhD Year 2006 Name of Author CHURCH
Andrew
John

Anti-basal ganglia antibodies in movement disorders

Thesis submitted for the degree of

Doctor of Philosophy

University of London

By

Mr. Andrew John Church

Neuroimmunology Department

Neuroinflammation

Institute of Neurology

University College London

December 2005

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

List of figures

List of tables

Abbreviations

Abbreviation	Definition
ABGA	Anti-basal ganglia antibodies
ASOT	Anti-streptolysin O titres
DNAse B	Deoxyribonuclease B
DTT	Dithiothreitol
ELISA	Enzyme linked immunoabsorbant assay
FITC	Fluorescein isothiocynate
GABHS	Group A beta haemolytic Streptococcus
HRP	Horse radish peroxidase
IF	Indirect Immunofluorescence
IgG	Immunoglobulin G
LDS	Lithium dodecyl sulphate
NSE	Neuron specific enolase
PAGE	Polyacrylamide gel electrophoresis
PANDAS	Paediatric autoimmune neuropsychiatric disorder associated with streptococcal infection
PNS	Paraneoplastic syndrome
RHF	Rheumatic fever
SC	Sydenham's chorea
SO	Streptolysin O
TS	Tourette's syndrome

Acknowledgments

For Sarah and Neve who this work is dedicated to, and thanks to Hugh, Diane, Eleanor and Paul.

A special thanks to Gavin Giovannoni and Ed Thompson for their encouragement and support. I would particularly like to thank Russell Dale, Paul Candler and Miles Chapman.

From the National Hospital for Neurology and Neurosurgery

Prof's Andrew Lees, Neil Quinn and Mary Robertson

From the Kennedy Institute of Rheumatology, Imperial College

Robin Wait

From the Institute of Child Health, Great Ormond Street Hospital

Robert Surtees, Isobel Heyman and John Hartley

This work was kindly funded by the University of London, Central Research Fund, Sophie Cameron Trust and the Tourette's society of America

Abstract

Sydenham's chorea (SC) is a neurological manifestation following group A Streptococcus infection (GABHS) and has been proposed as an antibody-mediated autoimmune disease. Other movement and psychiatric manifestations following GABHS have been recognised and termed Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS). It is proposed that PANDAS may be caused by the same antibody as SC. As the symptoms of PANDAS are identical to Tourette's syndrome (TS), the possibility that TS might turn out to be an autoimmune disorder has implications for the treatment and understanding of these disorders.

Evidence of GABHS was found in all patients with SC and PANDAS and 60% of patients with TS. Autoantibodies against basal ganglia (ABGA) were found in all acute SC and PANDAS patients. Only 25% of TS patients were ABGA positive. There was little evidence for ABGA in controls. There was a higher prevalence of ABGA in systemic diseases associated with GABHS but this did not reach significance.

ABGA bound to proteins with molecular weights (40, 45, 60 and 98 kDa) and these responses were variably found in SC, PANDAS and TS. The identification of these antigens proved to be problematic due to contamination with other proteins with the same molecular weights. Neurone specific enolase (NSE) was identified as one of the antigens. As this protein was not specific to basal ganglia it cast doubt as to the specificity of ABGA. Interestingly, however, enolase is also found on the surface of GABHS and has extensive homology with human enolase, thus lending support to the possibility of molecular mimicry derived autoimmunity.

1 Introduction

1.1 The aims of this introduction

- Examine the role of autoantibodies, cytokines and cellular responses in putative autoimmune syndromes.
- To examine neurological disorders thought to be immune or autoimmune mediated
- Focus on the role of group A streptococcus in immune-mediated disorders.
- To outline the clinical and scientific aspects of Rheumatic fever and its neurological manifestation, Sydenham's chorea
- To introduce the putative post-streptococcal disorder Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS) and its relationship to Tourette's syndrome
- Suggest how putative autoantigens can be identified using proteomic methods

1.2 Background to autoimmune diseases

Autoimmune diseases can affect individual organs such as the kidneys, liver, pancreas, thyroid, adrenal gland and perhaps brain (organ-specific autoimmunity) or affect multiple body systems simultaneously (non-organ specific autoimmunity), (Erman *et al.*, 2001). The causes for autoimmunity are complex and probably include genetic, environmental, infectious factors and perhaps several in combination. It is probable that aberrant lymphocyte responses and failure of tolerance (un-responsiveness to self antigens) is the major mechanism behind autoimmunity, but autoantibodies (antibodies against self-antigens), complement and cytokines are also important for disease induction and persistence.

1.3 Identification of autoantibodies in immune mediated disorders

In autoimmune syndromes, alterations in the immune system can result in the survival of autoreactive B lymphocytes which upon activation produce large amounts of autoantibodies which persist for years, such as anti-nuclear antibodies (Davidson *et al.*, 2001; Sato *et al.*, 1996). High levels of autoantibodies have been found in most autoimmune diseases, although their specific pathological role is frequently unclear. The exception has been the identification of the functional effects of autoantibodies in diseases such as myaesthenia gravis and the Lambert-Eaton myasthenia syndrome, where antibodies disrupt or block synaptic transmission of nerves (Vincent *et al.*, 1999 a; Whitney *et al.*, 1999). Graves disease, an autoimmune thyroiditis is also thought to be antibody- mediated, as the autoantibodies stimulate the thyroid hormone receptor,

causing dysfunction (Weetman., 2001). Apart from their role in autoimmunity, autoantibodies of low affinity are also produced as a consequence of infection, tissue trauma and tumours, but usually disappear after recovery and are probably a feature of inflammation and tissue repair (Dighiero *et al.*, 1999; Rose., 1998; Silverman., 1997).

The major use of autoantibodies is as a laboratory marker, as their detection is useful as a diagnostic tool when identifying a possible autoimmune syndrome. Results need to be interpreted in the context of clinical presentation, age of the patient (numbers of naturally-occurring autoantibodies increase with age) and other underlying conditions such as infection and tumours. Autoantibodies are typically detected in serum using indirect immunofluorescence, enzyme or radio-ligand-linked immunoabsorbant assays (ELISA), particle agglutination or Western immunoblotting (electrophoresis). The various assays have different sensitivities and specificities due to the presentation and concentration of antigens and the methods of visualising antibody binding (colorimetric, turbidimetric, fluorometric, enzymatic, radioactive or chemiluminescent). These differences are important when considering an autoantibody as a surrogate marker of autoimmunity.

1.4 Immune-mediated neurological syndromes

Apart from MS, which is considered to be the classic immune-mediated central nervous system (CNS) syndrome, paraneoplastic neurological syndromes (PNS) are the most widely studied of these CNS disorders (Dropcho., 1999; Sutton *et al.*, 2002). The pathophysiology of PNS involves the development of an immune response against a tumour outside the CNS, commonly small-cell lung (SCLC) or gynaecological tumours (Rees *et al.*, 2003). This immune response is targeted against CNS antigens expressed by the tumour (Rees *et al.*, 2003; Sutton *et al., 2002*). This leads to a cross-reactive immune response between the tumour and normal brain tissue resulting in the destruction of neurons via immune mechanisms, rather than through the secondary spread of the tumour. Neurological symptoms associated with PNS include encephalitis, ataxia and motor or sensory neuropathies (Dropcho., 1999; Scaravilli *et al., 1999;* Rees *et al.*, 2003; Sutton., 2002; Sutton *et al., 2002*). Most paraneoplastic syndromes cause progressive brain damage and have characteristically high mortality rates.

An important feature of PNS is the presence of anti-tumour antibodies that recognise CNS antigens (anti-neuronal antibodies), and include: anti- Hu, Yo, Ri, Ma or Tr amongst others (Dropcho., 1999; Inuzuka., 2000). In addition, antibodies can also be directed against neuronal ion channels including (voltage-gated calcium and potassium channels). The ion channel antibodies are thought to be directly pathogenic to neuronal function (Newson-Davis., 1999; Rees *et al.*, 2003). Anti-calcium channel antibodies are found in patients with the Lambert-Eaton myaesthenic syndrome (LEMS) which is associated with SCLC (Lang *et al.*, 2003; Newson-Davis., 1999; Rees *et al.*, 2003). IgG

from patients with LEMS has been shown to bind to surface P/Q type voltage-gated calcium channels and cause a reduction in cell calcium currents leading to a motor disorder (Lang *et al.*, 2003; Motomura *et al.*, 1997; Vincent A., 1999 b).

There is controversy however as to whether antibodies against CNS antigens such as Hu and Yo are an important mechanism in PNS or are simply an epiphenomen. Unlike ion channel antibodies, the antigens recognised by some anti-neuronal antibodies are intracellular, which casts doubt as to whether they are capable of damaging neurons or altering their function (Dropcho., 1999; Rees *et al.*, 2003; Sutton., 2002). Transfer of anti-neuronal antibodies into animal models has so far failed to replicate the neuronal destruction seen in PNS (Tanaka *et al.*, 1994; Tanaka *et al.*, 1995). Alternatively these antibodies may be produced following neuronal damage and loss. Once produced these antibodies could then act to continually prime or up-regulate the immune response, thereby maintaining a state of pathological autoimmunity. This has been suggested by a recent study showing that anti-neuronal antibodies can induce expression of adhesion molecules and neuronal differentiation (Tanaka *et al.*, 2004).

The alternative mechanism of immune mediated neuronal loss in PNS is via cytotoxic T-lymphocytes (CTL) which are directed primarily against the tumour but also recognise CNS antigens. This cell-mediated, anti-tumour response probably explains the small size of tumours associated with PNS (Rees *et al.*, 2003). Expanded populations of MHC class I restricted CTLs directed against a tumour antigen which cross-reacts with the brain antigen (cdr2) have been reported in the blood of patients with PNS and may contribute

to development of CNS autoimmune destruction (Albert *et al.*, 1998). Evidence from histological analysis of the most common paraneoplastic syndrome (Hu-positive encephalomyelitis associated with SCLC) showed a cellular inflammatory infiltration of the cerebellum with a preponderance of T lymphocytes clustered around neurons (Bernal *et al., 2002*). It has also been shown that the T lymphocytes associated with this paraneoplastic syndrome are predominantly cytotoxic (CD8+) T cells and can be activated by peptides from the common paraneoplastic antigens Hu and Yo (Tanaka *et al.*, 2001). Further work to examine cytotoxic neuronal damage is important to elucidate this hypothesis further. However it is becoming clear that a cellular response, rather than antibody activity is probably responsible for the damage in PNS, although anti-neuronal antibodies remain a useful diagnostic marker (Rees *et al.*, 2003; Sutton., 2002).

1.5 Molecular mimicry hypothesis of post-infectious immune-mediated disorders

In addition to a tumour-triggered autoimmune phenomenon, many investigators have argued the possibility that pathogens could trigger autoimmune or para-infectious immune-mediated diseases (Kirch., 1993; Levin *et al.*, 2002; Ruckenstein *et al.*, 1993; Tsunoda *et al.*, 2002). This has been termed the molecular mimicry theory of autoimmunity, although several factors including genetic, environmental and hormonal are probably required for induction of disease. There is some, albeit conflicting, evidence for a molecular mimicry mechanism in the peripheral nervous system diseases: Guillain Barrē syndrome (GBS) and acute inflammatory demyelinating polyneuropathy, which can be triggered by the bacterium *Campylobacter jejuni* (Rees *et al.*, 1995; Tsang *et al.*,

2002). It is normal for an infection to trigger an antibody response against the bacteria but, due to similarity in structure or sequence of bacterial antigens and nerve gangliosides, such as GM1 and GQ1b, antibody binding is thought to lead to conduction block in peripheral nerves (O'Hanlon *et al.*, 2001; Weber *et al.*, 2000).

Antibodies binding to the GM1 ganglioside have been shown to block the normal action of sodium channels of nerves in the presence of complement, supporting a possible pathological mechanism for conduction block (Weber *et al.*, 2000). However, a functional effect of anti-ganglioside antibodies on conduction is not always found. A recent study of twelve patients with GBS showed no effect on axonal conduction by anti-ganglioside antibodies (Dilley *et al.*, 2003). The variations in results may mean that other immune mediators such as cytokines, cells or the presence or absence of complement components are important in GBS. Alternatively the concentration of antibody may be associated with an increase of functional effects (Odaka *et al.*, 2003). The presence of anti-ganglioside antibodies alone is not sufficient to prove their role.

1.5.1 Viral associated autoimmune disease and molecular mimicry

Whilst viruses are proposed as a trigger for autoimmunity in many disorders including MS, the exact mechanisms of this are unclear, but using GBS as a model, molecular mimicry could be one likely cause. An alternative hypothesis for viral-induced neuronal damage is apoptosis of neurons as a consequence of infection or the immune response.

1.5.2 The Human T-cell Lymphotropic Virus type one: associated central nervous system disorders

One possible model for autoimmune CNS disorders is infection with the Human T-cell Lymphotropic Virus type one (HTLV-1). HTLV-1 infects CD4+ T-helper lymphocytes and can cause adult T cell leukaemia or lymphoma (Poiesz *et al.*, 2003). HTLV-1 infection can also lead to a CNS disorder: HTLV associated myelopathy/tropical spastic paraparesis (HAM/TSP), (Osame *et al.*, 1986; Levin *et al.*, 2002). HAM/TSP clinically resembles MS, featuring limb spasticity, bladder disturbance, muscle weakness and sensory disruption (Ishak *et al.*, 2002; Izumo *et al.*, 2000). These neurological symptoms are associated with demyelination and axonal loss of neurones which are predominantly located in the spinal tracts where there is an associated lymphocytic infiltration (Izumo *et al.*, 2000; Levin *et al.*, 2002; Moore G *et al.*, 1989).

An immune mediated cause has been proposed for HAM/TS (Levin *et al.*, 2002; Jernigan *et al.*, 2003) since cytotoxic T cells infected with HTLV-1 have been found in the CSF of patients with the condition (Nagai *et al.*, 2001). The viral load of these infected cells may be associated with disease severity (Matsuzaki *et al.*, 2001; Yamano *et al*, 2002). It is also known that HTLV-1 infected CD4+ lymphocytes become activated and can enter the CNS through interactions with the endothelium of the blood/brain barrier allowing activated CD8+ cytotoxic lymphocytes, antibodies and cytokines into the CNS (Giraudon *et al.*, 2000; Romero *et al.*, 2000). The cytotoxic CD8+ lymphocytic response in HAM/TSP has been shown to be specific for the N terminus of the HTLV-1 tax antigen (Jacobson *et al.*, 1990; Lai *et al.*, 1994). The tax antigen is similar to the human leukocyte

antigen-A2 (Jacobson *et al.*, 1990). It is possible that the infected T cells may be causing the immune-mediated destruction of neurones in HAM/TSP. Interestingly, the HTLV-1 tax antigen CTL response has also been proposed as a protective anti-viral response so that autoimmunity or protection might be a host-specific reaction rather than one induced by a virus (Bangham., 2000).

It has also been reported that antibodies reactive against the C terminus of the HTLV tax antigen are found in HAM/TSP (Jerningan *et al.*, 2003; Lai *et al.*, 1994; Levin *et al.*, 2002). These antibodies cross-react with the human nuclear, ribonuclear protein–A1 (hnRNP A), and IgG isolated from brain and CSF from HAM/TSP patients also binds to hnRNP A-antigen (Jernigan *et al.*, 2003; Levin *et al.*, 2002).These antibodies have been shown to react strongly with Betz cells (Levin *et al.*, 2002), which are an important cell in the spinal tract (Lassek., 1940). Whilst antibodies may be important in HAM/TSP, it is likely that a combination of the CTL and the antibody response is involved in neuronal dysfunction and loss.

1.6 Bacteria and autoimmunity

In addition to viruses, a number of other pathogens including bacteria have been associated with autoimmune disease. For example the spirochete, *Borrelia burgdorferi*, which is transmitted by tics, typically causes cutaneous disease, encephalitis and arthritis (Steere., 2001). *Borrelia burgdorferi sensu stricto* infection is usually associated with a reactive arthritis, but 10% of these patients also develop an autoimmune form of Lyme's disease arthritis. This form of arthritis is associated with constant inflammation and treatment resistance (Guerau-de-Arellano *et al.*, 2002; Steere., 2001).

A HLA marker, HLA-DRB1*0401 has been associated with susceptibility to this form of Lyme's disease arthritis, which may be an immune complication of infection with *Borrelia* species (Guerau-de-Arellano M *et al.*, 2002; Steere *et al* 1998). Lyme's disease arthritis is also associated with an immune response against a surface antigen of *Borrelia burgdorferi* (OspA) which is homologous to the human lymphocyte function antigen-1 (LFA-1), (Gross *et al.*, 1998; Steere *et al.*, 2003). It is not known why patients develop arthritis rather than having a disseminated or organ-specific autoimmune disorder. Infection with *Borrelia* has occasionally been associated with chorea (movement disorder) although the cause of this is unknown (Piccolo *et al.*, 1998). The common bacterial infection associated with movement disorders is thought to be the group A beta haemolytic streptococcal bacteria.

1.7 Group A beta haemolytic streptococcal bacteria

Beta-haemolytic streptococcal bacteria are frequent pathogens in humans causing pharyngitis, skin and soft tissue infections but also have the potential to cause severe complications such as septicaemia, toxic-shock syndrome, meningitis and post-infectious immune-mediated syndromes (Barnham., 1989; Bisno *et al.*, 2003; Cunningham., 2000; Keefer *et al.*, 1937; Stillerman *et al.*, 1961). The incidence of asymptomatic colonisation of the throat has been reported to be as high as 60% in school children although typical rates are more probably 7-30% (Quinn *et al.*, 1957; Cornfield., 1958). The incidence of infections and community outbreaks has been linked to overcrowding, poor sanitation and inadequate access to healthcare (Dagan *et al.*, 1987; Rushdy *et al.*, 1995; Quinn *et al.*, 1957).

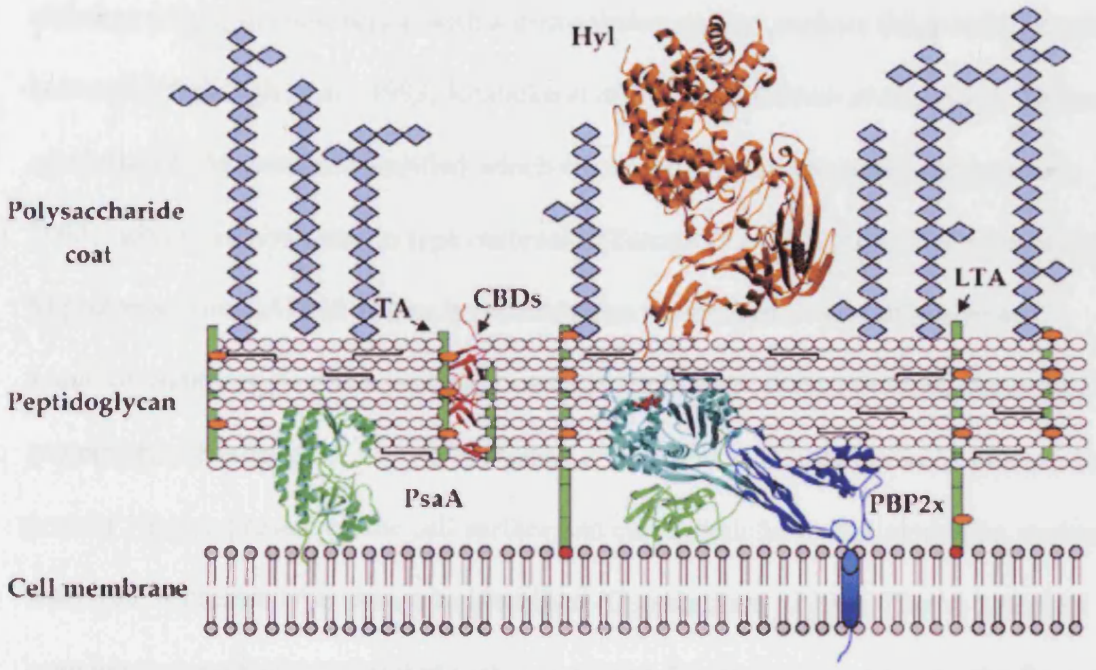
The original laboratory classification of different streptococcal species was based on the ability of cultured bacteria to cause different types of haemolysis when grown on blood containing agar plates. This was described as: alpha, (partial), beta, (clear) and no haemolysis, based on appearance and amount of haemolysis (Brown., 1919). Lancefield was the first to carry out serological grouping of the known streptococcal species based upon differences in the cell-wall polysaccharides, and designated them as types A-G (Lancefield., 1933; Lancefield., 1962). Thirteen streptococcal species have been identified to date, but *streptococcus pyogenes*, which is a gram positive, beta-haemolytic streptococcus and contains the cell-wall group A carbohydrate is considered to be the most important and frequent species causing human disease. However the other streptococcal species can also cause disease in humans, such as the group B streptococci

which are an important source of infection in neonates (Franciosi *et al.*, 1973). Group C and G streptococci have also been associated with pharyngitis and more rarely fasciitis, cellulitis and glomerulonephritis (Turner *et al.*, 1990; Efstratiou., 1989).

The cell wall of the group A beta haemolytic streptococcus (GABHS) consists of a peptidoglycan backbone with lipoteichoic acid components which provide structural stability and facilitate adherence to pharyngeal epithelial cells (Figure 1.1, Hasty *et al.*, 1992). The cell wall also contains the group A carbohydrate, which is composed of *N*-acetylglucosamine linked to a rhamnose polymer backbone. Streptococcal species can also be encapsulated but this varies according to the strain (Bisno *et al.*, 2003). Encapsulation of the bacterium influences infectivity and non-capsulated strains may be 100 fold less virulent than a capsulated bacterium (Wessels MR *et al.*, 1991). The cell wall additionally contains a number of proteins which increase adherence to epithelial cells and infer pathogenicity. For example streptococcus binds to fibronectin which enhances adherence to pharyngeal cells and this is mediated by a streptococcal binding protein (protein F), (Dinkla *et al.*, 2003; Okada *et al.*, 2003). Pathogenic strains which contain large amounts of protein F are more likely to cause invasive disease (Natanson *et al.*, 1995). Other proteins infer protection from the immune response such as the complement inhibitory protein, which is an extracellular protein and capable of inactivating the membrane attack complex which is the end-stage of the complement pathway (Akesson *et al.*, 1996).

Figure 1-1 diagrammatical representation of the Streptococcal cell wall proteins

http://www.nature.com/embor/journal/v3/n8/fig_tab/embor106_f2.html



Teichoic acids (TAs) and lipoteichoic acids (LTAs) (both in green) are carbohydrate phosphate polymers rich in choline (orange spheres). TAs are linked to the peptidoglycan via a phosphodiester linkage, whereas LTAs are linked to the cell membrane via a C-terminal fatty acyl group (in red). Choline-binding proteins are linked to cell-wall TAs or LTAs via choline-binding domains (CBDs) (red ribbon diagram). In Streptococcal Pneumococcal strains, a surface antigen (PsaA) is located underneath the peptidoglycan layer and is attached to the cell membrane via an LXXC motif; penicillin-binding proteins (PBPs) are located in the periplasmic space and interact with the peptidoglycan, and display a single, N-terminal transmembrane helix (in blue). Hyaluronate lyase (Hyl) is tethered to the peptidoglycan via an LPXTG motif.

Enolase is present in the cytoplasm and in small quantities on the cell wall; the mechanism for translocation to the cell surface is not known (Pancholi V, 1998 and 2001).

In addition to streptococcal identification and typing using differences in the cell wall carbohydrate, GABHS can also be sub-divided into serotypes due to variations in an important cell-wall protein, the M protein (Lancefield., 1962). The M protein is a coiled fibrillar protein, consisting of 4 regions of repeating amino acid sequences with a proline-rich area which, in conjunction with a hydrophobic region, anchors the protein into the cell-wall (Harbaugh *et al.*, 1993; Khandke *et al.*, 1990; Robinson *et al.*, 1992). There are currently 124 M proteins identified which are coded for by *emm* genes (Bisno *et al.*, 2003), which are now used to type outbreaks (Tanaka D *et al.*, 2002). The variability of M proteins from GABHS strains is probably due to iatrogenic recombination and acquired mutations so that a particular *emm* gene does not always produce the same M protein type (Harbaugh *et al.*, 1993; Penney *et al.*, 1995). In addition to M protein, the 'T protein', is also present on the cell surface and can be helpful in distinguishing strains when the M protein type cannot be identified (Cunningham., 2000). The M types and *emm* genes are usually associated with a particular T type thus determining the T type enables M protein identification to be carried out rapidly (Beall *et al.*, 1997; Beall *et al.*, 1998; Cunningham., 2000).

The M protein is conserved between strains carrying the same M type, except for 2 regions close to the N terminus which protrude from the cell and contains both variable and hypervariable regions (Fischetti *et al.*, 1989; Robinson *et al.*, 1992). Antibodies raised to the hypervariable region confer host protection from re-infection with GABHS carrying the same M protein, but not from other heterologous strains because of the antigenic variations in this region (Beachey *et al.*, 1981). Equally the M protein is an

important protective factor for the streptococcus as its presence confers anti-phagocytic properties and therefore protection from complement binding opsonisation and phagocytosis (Beachey *et al.*, 1980; Bisno *et al.*, 2003., Ehlenberger *et al.*, 1977; Jones *et al.*, 1988). The mechanism of protection from phagocytosis is attributed to the specific binding of serum complement factor H to the conserved M protein region along with fibrinogen, thereby preventing complement component C3b from being deposited on the surface of the streptococcus and causing inactivation of C3b (iC3b), (Berggard *et al.*, 2001; Bisno *et al.*, 2003; Fischetti., 1995; Horstmann *et al.*,1992). The importance of the protein is also underlined by its alternative function as an adhesion factor aiding attachment to pharyngeal epithelial cells and keratinocytes (Caparon *et al.*, 1991; Ellen *et al.*, 1974).

The function of the M protein variable regions however is poorly understood. These regions may confer pathogenicity and protection from the immune response again through interfering with complement deposition. Human complement inhibitory factors (C4BP and Factor H-like protein-1) both bind to the variable region of the M-protein and may also prevent phagocytosis by degrading complement component C3 (Berggard *et al.*, 2001; Johnsson *et al.*, 1998; Thern *et al.*, 1995). This may act in conjunction with Factor H binding to the conserved region of the M protein to protect virulent strains from complement-mediated immune attack (Johnsson *et al.*, 1998).

Streptococci also produce a number of extracellular pyogenic exotoxins (SPE: A, B, C and F amongst others) which are important as they cause direct tissue damage (Hallas.,

1985; Saouda *et al.*, 2001; Watson., 1960) by initiation of apoptotic pathways or disruption of mammalian cell-membranes. The example of this process is SPE-B produced by GABHS, which is a serine protease enzyme and has been shown to induce apoptosis in cell lines (Kuo *et al.*, 1999; Tsai *et al.*, 1999). SPE-B has been shown to cleave immunoglobulins, vitronectrin and fibronectin thereby protecting from immune attack and in turn damaging the basement membranes in an infected host, damaging tissue and facilitating invasion (Bisno *et al.*, 2003; Collin *et al.*, 2001).The exotoxins can also act as superantigens which activate the immune system through interactions with MHC class II and the Vβ portion of the T-cell receptor (Marrack *et al.*, 1990). This can non-specifically activate large numbers of lymphocytes which produce a host of pro-inflammatory cytokines including TNFα and interferon-γ which can directly damage cells (Scherer *et al.*, 1993; Leonard *et al.*, 1991).

The streptococcal exotoxins and their role as superantigens have been implicated in a number of streptococcal diseases including Kawasaki's syndrome, which is an acute febrile systemic vasculitis of children (Yarwood *et al.*, 2000; Yoshioka *et al.*, 2003). The exotoxin is thought to cause an inappropriate T-cell mediated response through clonal expansion of lymphocytes, vascular infiltration and localised inflammation (Jason *et al.*, 1997; Yarwood *et al.*, 2000).

Streptococci also produce streptokinase, which is an extracellular protein that can bind to plasminogen to activate plasmin (Damaschun *et al.*, 1992; Norstrand *et al.*, 1996). These aids bacterial spread and may be important in the development of post-streptococcal

glomerulonephritis (Norstrand *et al.*, 1996). The remaining proteins such as the streptolysins and deoxyribonucleases are also toxins, aid infectivity and are useful for diagnosis and these will be discussed later.

Recent evidence has shown that GABHS is capable of invading human cells and being internalised where it can form a reservoir for persistent infection or colonisation (Cue *et al.*, 1998; Facinelli *et al.*, 2001; Hagman *et al.*, 1999; LaPenta *et al.*, 1994). Internalisation is thought to require multiple interactions with M protein and a fibronectin-binding protein, SfbI (Cue *et al.*, 1998; Dombek *et al.*, 1999; Molinari *et al.*, 1997). It has been proposed that skin and pharyngeal GABHS isolates are more invasive than those that cause blood sepsis (Gladstone *et al.*, 2003). GABHS has been recovered from tonsils of patients with recurrent streptococcal infection and from asymptomatic carriers who were free of symptoms at the time of surgery (Osterlund *et al.*, 1995; Osterland *et al.*, 1997). Survival of GABHS within host immune cells may be an additional route of internalisation and a method of circumventing the host immune response. A murine model of GABHS skin infection found that viable GABHS could be obtained from mouse phagocytic cells after *in vitro* or during *in vivo* infection (Medina *et al.*, 2003). A study of phagocytosis of GABHS by human neutrophils showed that GABHS is capable of surviving and therefore chronically infecting cells (Staali *et al.*, 2003).

1.8 Streptococcal infections

Pharyngitis is the most common consequence of streptococcal infection and typically presents as an acute fever with sore throat and is commonly associated with headache, nausea and sometimes vomiting. The pharynx is reddened with enlarged tonsils which are commonly covered in purulent exudates. The tongue may be red and swollen ('strawberry tongue'), and palatal petechiae may also be seen (Wannamaker., 1984). The majority of individuals with pharyngitis recover without treatment but because of the risk of serious post-infectious complications, antibiotic treatment is indicated even in cases of suspected infection. The clinical differentiation from other causes of pharyngitis such as viruses can, however be difficult (Breese., 1977).

The development of a diffuse red rash on the skin in addition to the pharyngeal symptoms is an important sign of scarlet fever and although now rare, in the later part of the 19th century mortalities between 25-30% were not uncommon (Rotch., 1896). Death was caused by upper airway obstruction, meningitis or damage to the jugular vein or carotid artery (Rotch., 1896). Scarlet fever is now thought to result from infection with a strain of streptococcus producing the pyogenic exotoxin C, which can cause direct tissue damage (Hallas., 1985; Watson., 1960). Other streptococcal skin infections include erysipelas which presents as an abrupt fiery red rash with defined margins and intense pain (Bernard *et al.*, 1989) and impetigo which is characterised by single or multiple thick crusted lesions (Dajani *et al.*, 1972). Streptococcus is also an important cause of cellulitis (Bernard *et al.*, 1989; Darmstadt *et al.*, 1994) and can also cause septicaemia, meningitis, vaginitis and, rarely, necrotising fasciitis.

1.9 Anti-streptococcal antibodies as surrogate markers of infection

Due to the delayed clinical manifestations in some of the post-streptococcal syndromes identification (culturing) and serotyping of the GABHS is not always possible as the infection may have cleared. In addition to a clinical history of pharyngitis or skin infection, surrogate markers can be used instead to assess recent infection. Surrogate markers measure the concentrations of host antibody levels against the extracellular antigens of GABHS. Increases or decreases in these antibodies can give some indication as to the course of the infection (acute, chronic, relapse or recovery) and hence the indication for treatment.

1.9.1 Anti-streptolysin O titre

Streptolysin O (SO) is an oxygen-labile, thiol-activated cytolysin, produced by GABHS and causes broad haemolysis on blood agar plates (Stevens., 2000). Two forms exist with molecular weights between 50-70 kDa and contain active fragments (Alouf *et al.*, 1988; Bhakdi *et al.*, 1984). The action of SO is through binding of the toxin to cholesterol on cell membranes resulting in toxin-cholesterol aggregates which cause cell lysis through an osmotic mechanism (Bhakdi *et al.*, 1985; Palmer *et al.*, 1998; Sekiya *et al.*, 1993; Sekiya *et al.*, 1996). Streptolysin O is also capable of affecting the immune response by impairing neutrophil function and inducing monocytes to produce tumour necrosis factor (TNF) and keratinocytes to produce a host of inflammatory cytokines (Bremm *et al.*, 1987; Hackett *et al.*, 1993; Ruiz *et al.*, 1998).

Neutralising antibodies are produced by the host as a protective measure against SO and are called anti-streptolysin O antibodies (ASO), (Todd., 1932; Wannamaker., 1958). Antibody titres against SO (ASOT) rise during GABHS infection and usually reach a peak between 3 and 6 weeks after acute infection before falling (Todd., 1932). A raised ASOT is found in 80% of patients with acute pharyngitis, and rising titres are suggestive of a very recent infection (Special Writing Group of the Committee of Rheumatic Fever., 1992).The measurement of ASOT does have limitations, however, as streptococcal skin infections usually elicit a poor ASOT response (Kaplan *et al.*, 1974).

1.9.2 Anti-deoxyribonuclease B titre

Streptococcus also produces a number of deoxyribonucleases (DNAses A, B, C and D) which can break down DNA and also elicit an immune response (Ayoub *et al.*, 1962; Wannamaker., 1958). Due to the limitations of ASOT, measuring antibodies against DNAse is also a useful indicator of GABHS as it is raised in skin infections and is elevated for longer than ASOT (Ayoub *et al.*, 1962). Neutralising antibodies against DNAse B rather than A, C or D result in the strongest response during streptococcal infections (Wannamaker., 1959). The benefit of anti-DNAse B is that the antibody response is still detectable later on in the disease course compared to ASOT and is more pronounced (Tiesler *et al.*, 1976). Anti-DNAse titres are not thought to reach their maximum titre until 6 to 8 weeks after acute infection (Kaplan *et al.*, 1974). Other surrogate markers exist such as anti-hyaluronidase, although it closely mirrors that of anti-DNAse B and adds little to the diagnostic utility of the anti-DNAse B test.

ASOT and DNAse B titres are both measured in serum samples, usually by particle agglutination, enzyme-linked immunoassays or the increasingly common, rate nephelometry methods. A streptococcal serology result is considered raised if the level is above the upper-limit of normal for a population (Kaplan *et al.*, 1998). Recently it has been proposed that cut-off limits should be used in conjunction with age ranges, as raised streptococcal serology is usually undetectable in neonates and infants and mean normal values also fall in adults (Igari *et al.*, 1983; Klein *et al.*, 1971; Renneberg *et al.*, 1989). A World Heath Organization standard exists for ASOT (ASOT <200IU/mL), (Spaun *et al.*, 1961). A normal value for anti-DNAse B is considered by most UK laboratories to be <300 IU/mL and results are usually reported as international units per millilitre (IU/mL).

1.10 Post-streptococcal immune-mediated syndromes

There are a number of post-streptococcal, immune-mediated clinical syndromes which have been suggested as being autoimmune. They are typically latent or delayed manifestations of infection occurring weeks to months after acute infection and are thought to be caused by GABHS triggering a hyperimmune or autoimmune reaction. These syndromes include post-streptococcal glomerulonephritis (PSGN), vasculitides such as Kawasaki's syndrome, reactive arthritis (PSA), rheumatic fever (RHF) and Sydenham's chorea.

1.10.1 Post-streptococcal glomerulonephritis

Post-streptococcal glomerulonephritis (PSGN) usually occurs in a minority of people 3-6 weeks after infection with a nephritogenic strain of GABHS (Nissenson *et al.*, 1979). The

clinical presentation is variable but the most common features are: oedema, haematuria, back pain, malaise and disruption of normal kidney function (Norstrand *et al.*, 1999; White *et al.*, 2001).

The pathophysiology of PSGN has been studied in depth using histological methods which showed that there is an early infiltration of white blood cells with a preponderance of CD4+ lymphocytes, deposition of IgG and complement component C3 within the mesangium and capillaries of the kidney (Matsell *et al.*, 1994; Parra *et al.*, 1984). Initiation of this immune response may be triggered by deposition of erythrogenic toxin B within the glomeruli which triggers a local inflammatory reaction perhaps causing innocent bystander damage (Rincon *et al.*, 2003; Treser *et al.*, 1969). Alternatively M protein from GABHS has been identified as part of immune complexes found in the renal glomeruli of PSGN patients (Kantor *et al.*, 1965; Nordstrand *et al.*, 1996). Immune complexes are highly immunogenic and may cause secondary inflammation in the glomeruli resulting in impaired renal function. Nephritogenic strains of GABHS also produce streptokinase and proteinases which have been shown to deposit in and alter glomerular architecture (Cu *et al.*, 1998; Norstrand *et al.*, 1998; Nordstrand *et al.*, 1999; Peake *et al.*, 1991). It is possible that all these mechanism are involved in PSGN and each may be important in different stages of the disease (acute, chronic and relapses). The majority of the studies have all found that streptococcal proteins either on the cell surface or extracellular are important in the pathogenesis of PSGN.

In addition to T-lymphocyte responses and complement activation, antibodies may also be important in streptococcal immune mediated syndromes. Autoantibodies have been identified in post-streptococcal syndromes and may play a functional role. Antibodies directed against glomerular heparin sulphate proteoglycan have been shown to bind to mammalian and streptococcal hyaluronate, and have been reported in PSGN (Fillit *et al.*, 1985). Similarities in amino acid sequences and structure between streptococcal and human proteins have been described, such as the similarities between the coiled, coil structure of cardiac myosin, laminin and M protein (Cunningham *et al.*, 1993; Manjula *et al.*, 1985). These cross-reactive antibodies might serve to boost or prolong the immune response or may have a direct cytotoxic effect on tissue or, through the action of complement, cause localised inflammation and initiate an immune response.

1.10.2 Post-streptococcal arthritis

Arthritis is usually a major symptom of rheumatic fever (Special Writing Group of the Committee of Rheumatic fever., 1992) but isolated post-streptococcal, reactive arthritis affects young children and adolescents, causing pain and inflammation of joints and can relapse several times or lead to chronic arthritis (Ayoub *et al.*, 2000; Iglesias-Gamarra *et al.*, 2001).

1.11 Rheumatic Fever

Rheumatic fever (RHF) is a serious consequence of GABHS infection of the upper respiratory tract and there is a worldwide distribution of disease (Peter *et al.*, 1977; Smith *et al.*, 1993). The incidence of RHF has dramatically declined in developed countries since the 1940's probably due to better sanitation, healthcare and introduction of antibiotics. Nevertheless several outbreaks of RHF have occurred recently in western countries, underlying the importance of the disease and the differences in virulence between streptococcal strains (Bonora *et al.*, 1989; Veasy *et al.*, 1994; Wallace *et al.*, 1989). There is also evidence that susceptibility to RHF may have a genetic element since RHF occurs 4-8 times more frequently in relatives of patients who have had RHF (Read *et al.*, 1938; Wilson *et al.*, 1954). It has been suggested that familial susceptibility might be inferred by the presence of an autosomal recessive gene (Wilson *et al.*, 1954).

Later studies have suggested that genetic factors may be more important in the clinical manifestations of post-streptococcal disease than in susceptibility to RHF (Di Sciascio *et al.*, 1980; Gibofsky *et al.*, 1998). For example, in comparison with rates of RHF, arthritis and Sydenham's chorea (discussed later) were found to be more concordant in monozygotic than dizygotic twins (DiSciascio *et al.*, 1980). Attention has instead focused on finding a HLA trait marker for disease, but no single specific locus has yet emerged, although this may be related to population and ethnic differences. Attention has recently focused on a B-cell marker (D8/17) which was identified in patients with RHF (Herdy *et al.*, 1992; Khanna *et al.*, 1989; Taneja *et al.*, 1989; Zabriskie *et al.*, 1985). This marker

has been found in 100% of patients with RHF in one study but only 20-64% in another study, compared to 5-15% in controls (Herdy *et al.*, 1992; Khanna *et al.*, 1989).

RHF is an important model of the pathophysiology of post-streptococcal autoimmune syndromes because of the range of symptoms that are associated with it. Apart from familial or genetic traits, there may be an immune pre-disposition to developing RHF as people with innate immune hyper-responsiveness to vaccines and streptococcal antigens appear to be at greater risk for developing RHF or other post-streptococcal immune complications compared to controls (Ayoub *et al.*, 1980; Dudding., 1968; Meiselas *et al.*, Rejholec., 1957; Shulman *et al.*, 1974). It has been shown that those patients with high antibody responses against brucella vaccines are more likely to develop RHF compared to age/sex matched controls (Rejholec., 1957; Meiselas *et al.*, 1961). It has also been proposed that patients with RHF and valvular disease have higher antibody titres against a number of streptococcal antigens in comparison to patients with uncomplicated GABHS pharyngitis (Dudding *et al.*, 1968; Shulman *et al.*, 1974). This may support a tendency to immune hyper-responsiveness against the streptococcal bacteria, which could be linked to induction of post-streptococcal autoimmunity, due to failure of immune tolerance or regulation.

1.11.1 Diagnosis of rheumatic fever

Correct diagnosis of RHF is essential as it can cause permanent cardiac valvular damage, can recur after further infections leading to cumulative damage. Clinical criteria were developed in 1944 (Jones., 1944), (A high probability of diagnosis can be made using the

revised Jones criteria of 1992), (Special Writing Group of the Committee of Rheumatic fever., 1992). Diagnosis requires the fulfilment of 2 major criteria or 1 major and 2 minor criterions (Special Writing Group of the Committee of Rheumatic fever., 1992), (Table 1-1).

Table 1-1 The Jones criteria for diagnosing Rheumatic fever

Major Jones criteria	**Minor Jones criteria**
Arthritis	Fever
Carditis	Arthralgia
Chorea	Elevated acute phase proteins (CRP, ESR)
Erythema Marginatum	Abnormal EEG (prolonged PR interval)
Skin nodules	Evidence of GABHS (throat culture and/ or positive serology)

The major complication of RHF is carditis, which can lead to death in the acute stage (Special Writing Group of the Committee of Rheumatic fever., 1992). Carditis is usually associated with a murmur at the cardiac apex which indicates mitral valve involvement, which is the common site of cardiac inflammation. Pericarditis and congestive heart failure are relatively rare complications but can lead to sudden death. In addition to carditis, arthritis is a common manifestation and is associated with severe pain and progressively affects the major joints of the extremities (migratory polyarthritis), (Special Writing Group of the Committee of Rheumatic fever., 1992). The skin rash of RHF (erythema marginatum) is an early clinical manifestation which typically begins as bright pink spots on the trunk but sparing the face (Special Writing Group of the Committee of

Rheumatic fever., 1992). Painless subcutaneous nodules are very uncommon but present on the extensor surface of the major joints. The last major clinical criterion is chorea, which is a disorder of the central nervous system (CNS), and also termed Sydenham's chorea (Aron., *et al* 1965).

The minor RHF diagnostic criteria are fever, arthralgia and laboratory abnormalities such as elevated acute phase proteins (C-reactive protein and erythrocyte sedimentation rate), (Special Writing Group of the Committee of Rheumatic fever., 1992). A prolonged PR interval on electrocardiogram (ECG) shows interruption of normal cardiac function. Only 25% of RHF patients have a positive throat culture for GABHS at the time of diagnosis, so streptococcal serology tests are used as a surrogate marker of infection (Special Writing Group of the Committee of Rheumatic fever., 1992).

1.11.2 Immune system and Rheumatic fever

Apart from streptococcal antibody responses, autoantibodies directed against the heart have also been identified in the serum from patients with RHF using an indirect immunofluorescence technique (Cavelti., 1945). Immunoglobulins have also been found deposited in the cardiac tissue of patients who died from RHF suggesting a role for antibodies in pathogenesis (Kaplan *et al* 1964). These anti-myocardial antibodies have been shown to react against important structural components: myosin, tropomyosin and sarcolemmal sheath proteins (Dale JB *et al.*, 1982; Khanna *et al.*, 1992; Krisher *et al.*, 1985; Quinn A *et al.*, 1998).

Myosin has been considered to be the most important antigen in RHF due to the recognition of anti-myosin antibodies in patients with RHF and their cross-reactivity with the M protein on the streptococcal surface (Cunningham *et al.*, 1988; Cunningham *et al.*, 1989; Cunningham., 2003; Dale JB *et al.*, 1985). Immunisation of mice and rats against myosin has resulted in experimental myocarditis, confirming it is an important autoantigen in the pathogenesis of RHF, although the exact mechanism is unclear (Kodama *et al.*, 1991; Neu *et al.*, 1987). An amino acid sequence (Gln-Lys-Ser-Lys-Gly) has been identified that is common to both the structure of streptococcal M protein and myosin, and could possibly trigger the anti-myosin antibody response seen in RHF (Cunningham *et al.*, 1989). Interestingly the M protein, myosin and tropomyosin share a similar structure as all are coiled α-helical proteins (Cunningham *et al.*, 1993).

Mice immunized with streptococcal M proteins have been shown to produce antibodies which react against both myosin and tropomyosin (Fenderson *et al.*, 1989; Mertens *et al.*, 2000). Mice immunised with streptococcal M protein produce monoclonal antibodies that have been reported to be cytotoxic to cardiac cells but only in the presence of complement (Adderson *et al.*, 1998; Cunningham *et al.*, 1992). Anti-myocardial antibodies could therefore be directly cytotoxic; causing the tissue damage associated with RHF. However, transfer of the heart/streptococcal reactive antibodies into animal models has not always caused carditis (Smith *et al.*, 1993; Zabriskie., 1966; Zabriskie *et al.*, 1970). Anti-cardiac antibodies have also been reported following cardiac surgery so they are not unique to RHF (de Scheerder *et al.*, 1989). It is possible that anti-myosin antibodies are produced as a consequence of myosin being exposed from myocardial cells

that have been damaged by another process. This may be indicated by the presence of antibodies within myocardial cells as well as surrounding them (Kaplan *et al.*, 1964) and the production of myocardial autoantibodies following surgery (de Scheerder *et al.*, 1989).

The alternative role of anti-myocardial antibodies is to aid or perpetuate an autoimmune mediated, streptococcal driven CTL response against cardiac antigens. Studies of human monoclonal, anti-myocardial antibodies have found that in addition to myosin, autoantibodies also recognise the antigen: N-acetyl-β-D-glucosamine (GlcNAc), (Shikhman *et al.*, 1994). Affinity-purified human anti-myosin antibodies have been shown to react with GlcNAc and also cross-react with streptococcal M protein (Cunningham *et al.*, 1988). Interestingly it has been found that these antibodies also cross-react with connective tissue components, Vimentin and Keratin, suggesting they are not monospecific (Shikman *et al.*, 1993; Shikman *et al.*, 1994). A further study has also shown that a 'monoclonal' antibody reactive against myosin also recognises GlcNAc, is cytotoxic for endothelial cells but also reacts with the extracellular matrix protein, Laminin (Galvin *et al.*, 2002). This could mean that the antigenic epitope(s) recognised by the myocardial antibodies define cytotoxicity or the ability to affect the basement membrane and hence vasculature. Anti-myocardial antibodies may have an effect on the vascular endothelium causing damage and up-regulation of adhesion molecules which aid the recruitment of T-cells, because of the polyspecific moieties of the myocardial autoantibodies (Cunningham *et al.*, 1986; Cunningham., 2003; Galvin *et al.*, 2000;

Gulizia *et al.*, 1991). Therefore, it appears to be the common reactive antigen, myosin is not necessarily the most important pathologically.

Monoclonal anti-myosin antibodies raised in mouse models, especially monoclonal antibody (36.2.2) have, like the human antibodies, been shown to be reactive to endothelial antigens, including the vascular epitopes such as laminin (Antone *et al.*, 1997; Gulizia *et al.*, 1991). The polyspecificity of anti-myocardial antibodies may be an important mechanism in the pathogenesis of RHF as studying anti-myosin antibodies from patients with RHF and valvular disease showed that the antibodies also reacted with valvular endothelium. This affected vascular permeability and recruitment of immune mediators into the valve tissue (Galvin *et al.*, 2000; Gulizia *et al.*, 1991). Activation of the endothelium by these antibodies may aid cellular infiltration of the valve and expose myosin and other cardiac antigens to the immune system (Cunningham., 2003). Therefore antibodies may be directly pathogenic to cardiac cells in the early phase of the disease but are more important in aiding infiltration of CTLs and causing widespread damage such as valvulitis at a later stage.

In addition to antibodies, T-lymphocytes may be important and perhaps central to the pathogenesis of RHF. Patients with RHF have been reported to have heightened skin responses against streptococcal antigens when compared to controls and may show the importance of T-cells in the pathogenesis of RHF (Smith *et al.*,1993; Humphrey *et al.*, 1949). T cells from patients with RHF have been shown to be highly reactive against streptococcal antigens compared to T-cells from controls (Gray *et al.*, 1981; Guilherme *et*

al., 2001; Read *et al.*, 1974). Increases in streptococcal-activated CD4+ T lymphocytes have been reported by some in the peripheral blood of RHF patients, although others have shown a decrease in lymphocyte proliferation (Morris *et al.*, 1993; Bahr *et al.*, 1991). This discrepancy may be related to the fact that myocardial autoantibodies can transiently block lymphocyte proliferation (Bhatia *et al.*, 1989) so although antibodies may not be directly responsible for all the damage to heart tissue their presence certainly modulates the disease process.

Lymphocytes from patients with RHF have been shown to be cytotoxic to cardiac cells *in- vitro* (Friedman *et al.*, 1971; Yang *et al.*, 1977), as has stimulation of peripheral T cells with M protein (Dale *et al.*, 1987). Transfer of an experimental myocarditis-like disease into animals has also been achieved through the transfer of T-lymphocytes that were reactive to heart and streptococcal antigens and probably caused CTL mediated cellular damage (Neu *et al.*, 1990; Smith *et al.*, 1993; Quinn *et al.*, 2001). Only a few studies have investigated whether CTL isolated from human RHF patients are specific against myosin, other cardiac antigens or streptococcal epitopes (Guilherme *et al.*, 1995; Pruksakorn *et al.*, 1994). A link between streptococcus and the CTL response has only been reported in studies in RHF cardiac valvular disease, either through T-cells isolated from valve tissue of RHF patients (Guilherme *et al.*, 1995) or a study showing reactivity of myosin activated T-cells to streptococcal M protein-5 (Galvin *et al.*, 2002). Isolated T-cells clones have also been found to be reactive to the same M-5 protein of the streptococcus, which is of a serotype, associated with RHF (Guilherme *et al.*, 1995). M

protein has also been proposed as a superantigen for T-cells, perhaps activating cells non-specifically via the V-beta portion of the T-cell receptor (Watanabe-Ohnishi *et al.*, 1994).

Further evidence of the importance of cell-mediated pathogenesis in RHF comes from histopathological studies of hearts from patients who have died from RHF. An infiltration of lymphocytes, macrophages and mast cells, although predominantly CD4+ lymphocytic infiltrates, have been reported in RHF (Marboe *et al.*, 1985; Raizada *et al.*, 1983). Histological studies have also shown an increase in HLA-DR expression on both the infiltrating leucocytes and the surrounding vascular endothelium (Kemeny *et al.*, 1989). However, the exact relationship between streptococcal infection, antibody production, CTL response and autoantigens remains uncertain.

1.11.3 Detection of anti-myocardial antibodies in Rheumatic fever

Despite the controversy surrounding the pathological role of anti-myocardial antibodies they are a useful immunodiagnostic tool in RHF. The standard method for detecting anti-myocardial antibodies is IF (Cavelti., 1945). Fluorescent staining produces a characteristic appearance of striations in the presence of IgG or IgM autoantibodies. Anti-myocardial antibodies which react against myosin have been reported in RHF but the presence of these antibodies in neurological manifestations of GABHS has not been studied.

1.12 Neurological manifestations of streptococcal infections

The neurological manifestations of GABHS infection include meningitis, meningo-encephalitis and acute disseminated encephalitis (van de Beek D *et al.*, 2002; Pergami *et al.*, 1996). These are rare complications and mainly effect neonates, young children or adults with immune-deficiencies.

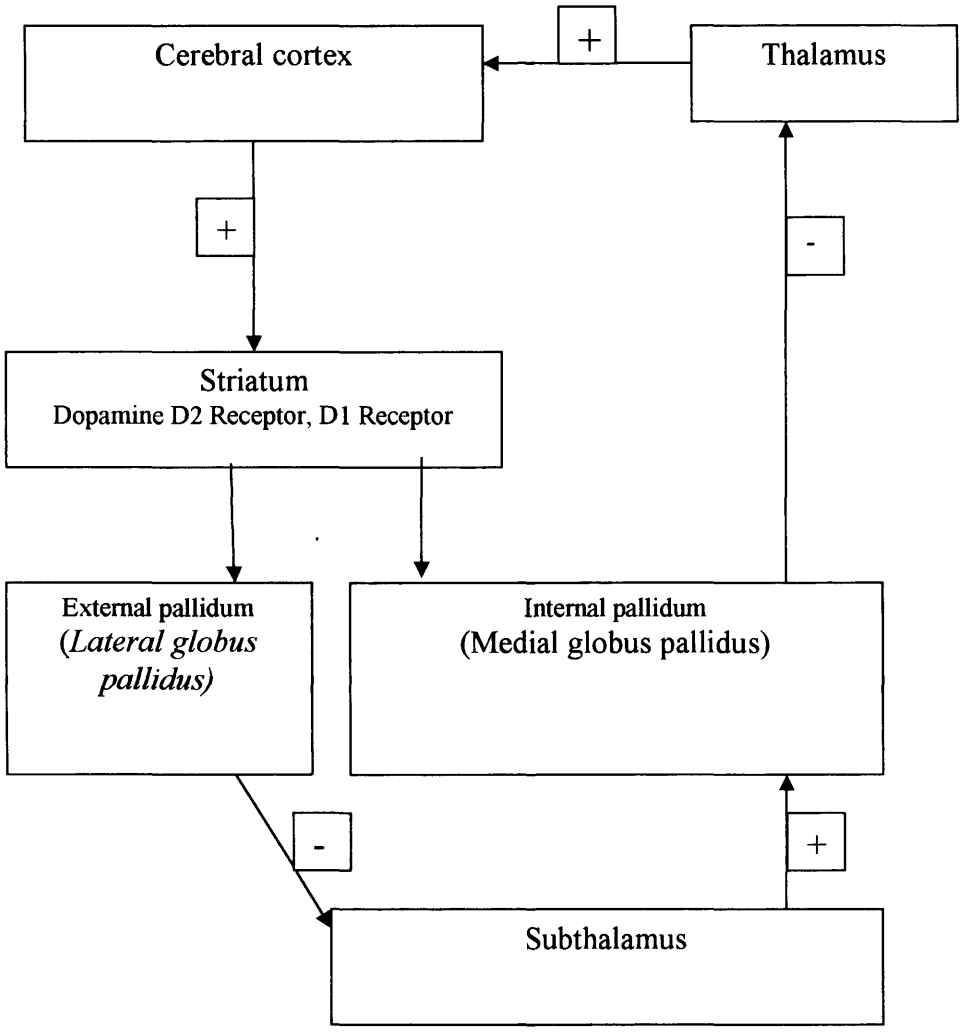
The classical neurological manifestation is Sydenham's chorea (SC) which is an acute onset movement disorder resulting from involvement of an area of the brain, the striatum, which controls movement and influences mood.

1.13 The striatum (basal ganglia)

The basal ganglia actually refer to a collection of nuclei which are located in the centre of the brain (deep grey matter). They include the caudate nucleus, putamen, globus pallidus and the subthalamic nucleus (part of the thalamus) and are collectively referred to as the striatum. The basal ganglia have been considered an important area in motor control of musculature since the 19th century (Ferrier., 1876). In addition to motor function the basal ganglia are also involved in emotional, behavioural and cognitive functions (Alexander *et al.*, 1986; Brown *et al.*, 1999; Nakano., 2000; Rolls., 1994).The basal ganglia do not have direct output connections to the spinal cord so the motor functions are mediated through the pyramidal system of neurones (Percheron *et al.*, 1994), (Fig 1-2).

Figure 1-2 Direct and indirect pathways through the basal ganglia, resulting (under non-pathological states) in a balance between excitation and inhibition

(Graybiel, 2001)



The neurons comprising the basal ganglia are arranged in a complex system but the efferent neurons are GABAergic medium spiny neurones (Parent *et al.*, 1993). These neurones contain the inhibitory neurotransmitter gamma-amino-butyric acid (GABA) and can be typed depending on the dopamine receptor type they contain (Albin *et al.*, 1989; Ribak *et al.*, 1979). Neuronal function within the basal ganglia is modulated by

dopaminergic innervations from both within and outside the striatum, especially from the substantia nigra (Cossete *et al.*, 1999). The main transmission system of the basal ganglia originates in the neocortex and the basal ganglia receive inputs from the associative areas of the neocortex and sensori-motor cortex (Gerfen., 1984; Haber *et al.*, 1995). The cortex utilises glutamate as the neurotransmitter and interact with the medium spiny neurones in the striatum (Bouyer *et al.*, 1984; Cherubini *et al.*, 1998).

The consequences of disorders affecting the basal ganglia are not completely understood but include hypo and hyperkinetic movement abnormalities and a range of neuropsychiatric symptoms. The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has given some insights into the relationship of the basal ganglia to Parkinson's disease (PD), a classical hypokinetic movement disorder characterised by rigidity and slow movements. MPTP causes extensive neuronal dopaminergic degeneration and a Parkinsonian phenotype due to over-reactivity of the neurones in the striatum and dysregulation of dopaminergic neurones (Herrero *et al.*, 1993).

Overt damage to the basal ganglia resulting in tissue lesions results from infections, tumours, vascular disorders, genetic disorders and trauma. Such lesions commonly result in apathy and loss of self initiative (abulia). The movement disorder dystonia, an extrapyramidal movement disorder with slow and sustained muscle contractions, is also common (Bhatia *et al.*, 1994; Fahn., 1988). The other common outcomes of basal ganglia lesions are chorea (fast, purposeless movements-hyperkinetic disorder) and athetoid-type

movement disorder (slow, sinuous movements), (Destée *et al.*, 1990; Denny-Brown., 1968). Diseases of the basal ganglia associated with progressive neuronal loss and a genetic pathogenesis or association, include Huntington's disease. This is typically a progressive hyperkinetic movement disorder with associated cognitive loss and dementia (Graveland *et al.*, 1985).Wilson's disease, involving abnormal copper metabolism, is another genetic disorder associated with degeneration of the putamen and globus pallidus causing tremor and rigidity (Giagheddu *et al.*, 2001; Wilson., 1912).

The basal ganglia are also involved in behavioural and emotional processing (Alexander *et al.*, 1986; Brown *et al.*, 1999; Nakano., 2000; Rolls., 1994). Lesions in the basal ganglia have also been related to the clinical onset of abulia, depression, disinhibition and confusion (Bhatia *et al.*, 1994). Psychiatric symptoms such as obsessive-compulsive and behavioural abnormalities have also been described associated with basal ganglia lesions (Laplane *et al.*, 1989). The reason for the psychiatric abnormalities is that basal ganglia circuits have functional roles in occulomotor, pre-frontal, and cingulate pathways which are central to attention, learning and behavioural control (Brown *et al.*, 1999; Gerfen., 1984; Haber *et al.*, 1995; Yamashiro *et al.*, 1997). Disruption in these circuits can therefore result in a range of extrapyramidal movement disorders and neuropsychiatric symptoms of which Sydenham's chorea is a good example. This disorder has been proposed as having an immune mediated pathogenesis.

1.14 Sydenham's chorea

Sir Thomas Sydenham described the extrapyramidal movement disorder (chorea) named after him in 1686 (Sydenham., 1848). Chorea was described as 'unsteadiness and convulsions of movements' which mainly affected the arms and legs. The movements were reported to be difficult to control or stop, and distinguished the disease from the form of religious dancing mania prevalent at the time (St Vitus dance) (Dale., 2003; Jummani *et al.*, 2001). There was speculation until the 19th century that Sydenham's chorea (SC) was the same as Huntington's disease (HD) which also presents with chorea, but they are now recognised as clearly different diseases. (Aron *et al.*, 1965; Dale *et al.*, 2003; Jummani *et al.*, 2001). It was not until the 19th century however that a link between infection and SC was made, with the discovery that a large number of patients with SC had rheumatic fever (Bouteille., 1810). Epidemiological studies have confirmed the relationship between GABHS infection, RHF and SC (Aron *et al.*, 1965; Ayoub *et al.*, 1966; Taranta *et al.*, 1956). SC is a major diagnostic feature of RHF (Jones., 1935; Special Writing Group of the Committee of Rheumatic fever., 1992) and may be a complication of RHF or a separate syndrome.

In common with other streptococcal pathologies there appears to be a limited number of strains associated with most cases of SC, typically M5, M6, M19 and M24 (Bronze *et al.*, 1993), although other strains or groups may be involved. Throat culture for GABHS is positive in 25%- 40% of patients with SC (Special Writing Group of the Committee of Rheumatic fever., 1992; Moore., 1996). Serotyping and strain tracking of GABHS infection(s) is complicated due to the latency of SC and RHF, which makes isolating the

organism in culture difficult (Taranta *et al.*, 1956; Cardoso *et al.*, 1993; Swedo *et al.*, 1993). The latency also means that other laboratory evidence of GABHS infection such as serology is not always positive. For example ASOT has been reported to be raised in 18-100% of SC patients depending on the study (Ayoub *et al.*, 1966; Eshel *et al.*, 1993; Moore., 1996). Other surrogate markers of inflammation such as the erythrocyte sedimentation rate and C-reactive protein can also be normal (Ayoub *et al.*, 1966; Special Writing Group of the Committee of Rheumatic fever., 1992; Shiffman., 1995).

SC is now considered to be a classic CNS manifestation occurring post-GABHS infection and is one of the major Jones criteria for the clinical diagnosis of RHF (Jones., 1945; Special Writing Group of the Committee of Rheumatic fever., 1992). SC is reported to occur in 20-30% of patients with RHF and is usually latent, occurring up to 6 months after the acute infection, but may sometimes be the primary diagnostic symptom of RHF (Aron *et al.*, 1965; Cardoso *et al.*, 1997; Swedo *et al.*, 1993; Terreri *et al.*, 2002). SC is more common in females than males (Aron *et al.*, 1965; Money., 1883; Swedo *et al.*, 1993) and most patients are children, below 18 years of age (Moore., 1996; Thayer *et al.*, 1906). Adult onset of SC is comparatively rare and most of the adult cases are associated with exacerbation of chorea following childhood SC.

SC is characterised by the acute onset (sometimes a few hours) of motor symptoms, classically chorea, usually affecting all limbs. Other motor symptoms include facial grimacing, hypotonia, loss of fine motor control and a gait disturbance (Aron *et al.*, 1965; Creak *et al.*, 1935; Kerbeshian *et al.*, 1990; Mercadante *et al.*, 2000; Swedo *et al.*, 1993). Fifty percent of patients with acute SC spontaneously recover between 2 to 6 months

(Cardoso *et al.*, 1993; Swedo *et al.*, 1993). Mild or moderate chorea or other motor symptoms can persist for up to and over 2 years in some cases (Cardoso *et al.*, 1993; Gibb *et al.*, 1985)

Recurrences of SC in childhood have been reported with the relapse rate reaching as high as 59% after 29 years of follow up in 1 study (Jones *et al.*, 1935). Re-exacerbation of chorea has also been associated with pregnancy (chorea gravidarum) with the majority of cases having had a previous history of SC (Beresford *et al.*, 1950; Cardoso., 2002). This may be influenced by infection, host susceptibility or more likely alterations in hormonal networks during pregnancy (Cardoso *et al.*, 1993; Cardoso *et al.*, 2002). Residual dopaminergic sensitivity following SC has also been reported (Nausieda *et al.*, 1983). This may indicate a potential cause of relapsing SC and chorea gravidarum, due to neuronal damage which occurred in the acute disease. This residual damage could manifest as chorea as a consequence of ageing. Re-exacerbation of SC has been reported to occur despite penicillin prophylaxis (Terreri *et al.*, 2002). This may also indicate the presence of subtle neuronal damage or perhaps failure of penicillin to protect from further streptococcal infections and recurrences of RHF (Murphy *et al.*, 2000).

Psychiatric manifestations are also common in SC (Freeman *et al.*, 1965; Moore., 1996; Swedo *et al.*, 1993). In one study 75% of patients were found to have co-morbid acute onset of obsessive-compulsive disorder (OCD), anxiety, depression and disruptive behaviour which persisted after resolution of the motor symptoms (Freeman *et al.*, 1965). Recent studies have suggested that OCD is the most common psychiatric

finding in SC (Swedo *et al.*, 1989) and an increasingly common presentation in those patients with relapsing SC (Asbahr *et al.*, 1999). A recent study looked at patients with RHF without chorea and found a high incidence of psychiatric symptoms including OCD (Mercadante *et al.*, 2000). The prevalence of OCD in RHF and acute and relapsing SC could therefore have implications in understanding the pathogenesis of idiopathic OCD and other emotional disorders and supports a pathophysiological link between RHF and SC.

Due to the rapid treatment of pharyngitis in Western countries with penicillin to prevent post-streptococcal complications, SC has become rare, although it is still a common cause of chorea and heart disease in children in developing countries (Cardoso *et al.*, 1993; Cardoso *et al.*, 1997; Kulkarni *et al.*, 1996; Swedo *et al.*, 1993). However, increases in the incidence of SC have also been reported unrelated to changes in healthcare (Goldberg *et al.*, 1993). Outbreaks of SC have recently been reported in developed countries even amongst communities with good access to healthcare, although this worldwide increase could be coincidental, it could also, suggest the emergence of highly pathogenic or antibiotic resistant strains (Ayoub., 1992).

1.14.1 Pathology studies of Sydenham's chorea brains

Due to the non-fatal course of SC, pathological studies of brain abnormalities have been rare and those that exist may only reflect severe or complicated cases, or inadvertently include cases of encephalitis, metabolic or genetic syndromes. The pathology reports all found abnormalities mostly localised in the basal ganglia, which included cellular

infiltration and neuronal loss with relative sparing of other brain areas (Colony *et al.*, 1956; Marie *et al.*, 1920), (Table 1-2). These focal changes have also been reported in the context of diffuse neuronal loss, which was the predominant feature, and an encephalitic pathogenesis was proposed (Greenfield *et al.*, 1922), (Table 1-2). The consistent findings appeared to be specific abnormalities of the basal ganglia with conflicting evidence regarding disseminated brain pathogenesis (Table 1-2). The clinical similarities of SC to HD may have influenced early reports of degenerative changes in SC brains (Table 1-2). However the similarities of SC to HD and the recognition of the basal ganglia as an area controlling movement, lead to the hypothesis that the basal ganglia were also the central area of pathogenesis causing SC (Aron., 1965; Jummani *et al.*, 2001; Dale RC., 2003).

Table 1-2 Pathological reports in Sydenham's chorea

Reference	Pathology	Conclusions
Delcourt, Sand, 1908	Inflammatory	Perivascular inflammation of basal ganglia and cortex
Guizzetti, Camisa, 1911	Inflammatory & vascular	Disseminated encephalitis
Harvier, Levaditi, 1920	Inflammatory	Perivascular inflammation of mesencephalon
Marie, Treitiakoff 1920	Inflammatory	Perivascular inflammation of basal ganglia
Greenfield, Wolfsohn, 1922	Inflammatory	Perivascular inflammation of basal ganglia and cortex
Lewy *et al.*, 1923	Inflammatory	Widespread
Ziegler, 1927	Degenerative	Basal ganglia
Lhermitte, Pagniez, 1930	Inflammatory/degenerative	Basal ganglia
Von Santha, 1932	Vascular	Encephalitic
Glaser, 1952	Vascular	Inflammatory features
Colony *et al.*, 1956	?Degenerative	Cortex & Thalamic involvement

1.14.2 Imaging studies in Sydenham's chorea

Brain imaging studies have been reported as normal in most cases of SC, casting doubt as to whether widespread neuronal loss is an important feature of the disease, although this does not rule out subtle alterations in neuronal function (Dale RC., 2003; Giedd *et al.*, 1995; Swedo *et al.*, 1993). Only rarely have suspected inflammatory changes seen on MRI, been associated with SC, and these have been predominantly localised to the basal ganglia (Castillo *et al.*, 1999; Kienzle *et al.*, 1991; Robertson *et al.*, 2002). These cases of SC have been shown to be reversible with disease remission, suggesting that temporary neuronal disruption rather than neuronal loss is a likely mechanism of pathogenesis (Giedd *et al.*, 1996; Trail *et al.*, 1995). One study reported an increased association between MRI and basal ganglia abnormalities, in SC patients who had repeated episodes of chorea during a 1-year study (Faustino *et al.*, 2003). The reports of MRI lesions in some cases of SC may be linked to severe disease spectrum or a tendency of SC to recur or become persistent in some patients.

Volumetric imaging studies have also confirmed basal ganglia (caudate nucleus and putamen) involvement in SC. The basal ganglia have been reported to be enlarged during acute SC compared to controls which may suggest inflammation (Giedd *et al.*, 1995). Further evidence for basal ganglia involvement in SC has come from magnetic resonance spectroscopy studies which have shown increased glucose turnover and hypermetabolism, which could suggest that alterations in local metabolism are important

(Goldman *et al.*, 1993; Lee *et al.*, 1999; Weindl *et al.*, 1993). It has been shown that these metabolic changes can be reversible with disease recovery which may be important in light of the reversible volumetric changes (Goldman *et al.*, 1993).

1.15 Pathogenesis of Sydenham's chorea

The pathogenesis of SC has shown some similarity to RHF in that SC has also been associated with autoantibodies that react both with rheumatogenic strains of streptococci and human basal ganglia (Husby *et al.*, 1976). The autoantibody is thought to derive from an immune response to streptococcal M or other surface protein, which cross-reacts with shared epitope(s) in the basal ganglia (Bronze *et al.*, 1993). The basal ganglia antibody response was removed by incubating positive serum with streptococcal membranes (Husby *et al.*, 1976). SC has therefore been proposed as another example of autoimmune molecular mimicry. The reactive basal ganglia and streptococcal antigens have not been identified so any functional or cytotoxic effects are unknown. Initially an IF method using human caudate/putamen frozen sections was used for detecting basal ganglia antibodies in SC (Husby *et al.*, 1976; Kiessling *et al.*, 1993; Kotby *et al.*, 1998). The original description of anti-basal ganglia antibodies (ABGA) reported antibodies that reacted against both caudate and subthalamic nuclei in 46% of patients with SC (Husby *et al.*, 1976), whilst a later report found 100% positivity in acute and 93% in chronic SC patients (Kotby *et al.*, 1998). Due to the inconsistency of these results and the small percentage of patients with ABGA in 1 study, the role of antibodies in an autoimmune SC model is still unclear.

The latency of disease onset in both RHF and SC supports an autoantibody-mediated pathogenesis; where as an immune-mediated cellular response would be more likely to result in a rapid onset of symptoms. Unfortunately no cellular studies have been reported in SC to date. One study has looked at genetic predisposition to SC but, like RHF, no HLA class I and II locus predicted susceptibility to disease (Donadi *et al.*, 2000). In common with RHF the B-cell marker D8/17 has also been proposed as a susceptibility marker in SC but the identity and function of the marker is unknown (Gibofsky *et al.*, 1998; Khanna *et al.*, 1989). Further evidence for an immune pathogenesis comes from case studies which indicate treatment with immune modulating drugs such as intravenous immunoglobulins (IVIG) and steroids are beneficial as they have been shown to decrease symptoms (Green., 1978; Cardoso *et al* 2003). Identification of the autoreactive antigens involved in SC would provide insights into the pathological effect of ABGA on the normal function of the basal ganglia circuits. This would allow for improved diagnostic tests and monitoring during treatment with immuno-modulating therapies, as antibody levels may decline with clinical recovery.

1.16 Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections

As neuropsychiatric symptoms occur commonly in association with SC, it has been proposed that disruption to basal ganglia circuits may result in primary psychiatric manifestations in addition to the movement disorder commonly seen (Moore., 1996; Swedo *et al.*, 1989). It was proposed that there was a possible relationship between outbreaks of streptococcal infections and a subsequent increase in children reported with acute onset of OCD, attention deficit hyperactivity disorder (ADHD) behavioural abnormalities and motor tics (Kiessling *et al.*, 1993; Swedo *et al.*, 1998). Dramatic symptom exacerbations were reported in these children following further streptococcal infections and recovery of symptoms appeared to mirror that of recovery from infection (Swedo *et al.*, 1998). None of the children had evidence of RHF or SC. Subsequently a clinical classification was devised: **P**aediatric **A**utoimmune **N**europsychiatric **D**isorders **A**ssociated with **S**treptococcal infections (PANDAS), (Swedo *et al.*, 1998). It was reported that PANDAS are phenotypically similar if not identical to Tourette's syndrome (Swedo *et al.*, 1998). However the clinical concept of PANDAS remains controversial (Kurlan., 1998).

The PANDAS classification is defined as the presence of OCD and/or a tic disorder which meets DSM-III-R or DSM-IV criteria with an acute, paediatric (pre-pubescent) onset occurring after 3 years of age, with a later episodic course of symptom exacerbations and recovery (Swedo *et al.*, 1998). The association with streptococcal

infection(s) was shown by a positive GABHS throat culture with initial raised streptococcal serology which declined with clinical recovery (Swedo *et al.*, 1989). Patients with RHF, SC or other neurological disease were excluded from the study in order to meet the clinical diagnosis of PANDAS (Swedo *et al.*, 1998). As a large number of patients are excluded by the narrow definition of the PANDAS classification, the phenotypic breadth of neuropsychiatric and motor disorder symptoms associated with streptococcal infections is currently unknown. In addition to PANDAS (OCD and Tics), a clinical description of 3 proposed PANDAS patients with anorexia as the major neuropsychiatric presentation has been reported (Sokol., 2000). Recently 2 adult cases which conform to a wider definition of PANDAS have been described, expanding the proposed syndrome classification (Bodner *et al.*, 2001; Martinelli *et al.*, 2002).

A link between the pathophysiology of SC and PANDAS has been suggested because of the temporal association between streptococcal infection and similar neuropsychiatric manifestations (Swedo., 1994). A further link between SC and PANDAS has been proposed due to the presence of ABGA in PANDAS. To date, this has only been investigated in 1 study, which reported that 44% of patients conforming to the PANDAS criteria, with childhood OCD and tics were ABGA positive (Kiessling *et al.*, 1993). Imaging studies have also suggested that PANDAS in common with SC is also associated with volumetric enlargement of the basal ganglia, particularly the caudate and putamen with sparing of other brain areas (Giedd *et al.*, 2000; Peterson *et al.*, 2000). Two studies have investigated the effects of infusing antibodies from patients with PANDAS into rat striatum with both reporting an increase in stereotypical movements (Hallett *et al.*, 2000;

Taylor *et al.*, 2002). However, another group have failed to reproduce this work (Singer *et al.*, 2003). The B-cell marker, D8/17 associated with RHF and SC has been reported in PANDAS using IF (Murphy *et al.*, 1997; Swedo *et al.*, 1997). Using a more specific flow cytometric analysis, D8/17 was also found to be increased but there was considerable overlap with controls casting some doubt as to its specificity and hence its ability to identify patients with these disorders (Hoekstra *et al.*, 2001).

Further evidence of an immune pathogenesis for PANDAS has come from the results of an IVIG and plasma exchange treatment study which showed a decrease in neuropsychiatric symptoms with treatment compared to controls, (Perlmutter *et al.*, 1999). Treatment studies have also investigated the use of antibiotics to clear the streptococcal infection or prevent further infections and symptom exacerbations. A double blind study using penicillin prophylaxis failed to show clinical benefit (Garvey *et al.*, 1999). Unfortunately compliance was reported to be a problem in this study. A prospective study of antibiotic use did demonstrate a clinical improvement in OCD symptoms (Murphy *et al.*, 2002). Unfortunately this was a descriptive study (Murphy *et al.*, 2002) so further appropriately controlled blinded studies are required. The potential of GABHS to reside within epithelial cells will influence the choice of antibiotics, dose and duration of treatment when conducting studies of this nature.

1.17 Tourette's syndrome

Georges Gilles de la Tourette gave his name to a syndrome which was first described in nine patients with a chronic tic disorder, neuropsychiatric and behavioral symptoms Tourette's syndrome (TS) is a neuropsychiatric disease characterised by chronic motor tics in addition to 1 or more vocal tics, which begins in childhood and persists in some cases to adulthood (Jancovic., 2001; Robertson., 2000). The prevalence of TS is thought to be 5 per 10,000 and is much higher in males (3-1, M: F ratio), (Hornsey *et al.*, 2001; Kadesjo *et al.*, 2000; Kurlan *et al.*, 2001*)*. Tics begin in childhood, occur several times a day and for a diagnosis of TS must persist for greater than 1 year although fluctuations in the severity and presiding type of tic can also occur over several years (Leckman *et al.*, 1998; Robertson., 2000). TS is ubiquitous throughout the world and its expression is not significantly different between cultural or socio-economic groups (Robertson., 2000).

A common feature of TS is the presence of co-morbid neuropsychiatric symptoms including OCD, ADHD, anxiety and depression (Jankovic., 2000; Kurlan *et al.*, 2001; Robertson., 2000). As with motor symptoms, neuropsychiatric symptoms vary in severity and type over time, a typical waxing and waning course being the classic feature of TS. The clinical course is typically of a childhood onset (mean age 7 years old) and whilst most children have complete or partial resolution of the symptoms on reaching adulthood, some patients will have persistent disease (Robertson., 2000). The reason for this is not currently understood, but may reflect a different pathogenesis or a particular phenotype indicated by permanent brain alterations.

Large family studies have suggested that TS is, at least partly, genetically determined, (Singer., 2000; Robertson., 2000). Early reports supported an autosomal dominant inheritance although no gene has yet been identified (Eapen *et al.*, 1994; Kurlan *et al.*, 1986; Price *et al.*, 1985). A systematic whole-genome screen has revealed 2 regions, 4q and 8p, with increased lod scores. These loci might yet reveal TS-related susceptibility genes (The TS Association., 1999). However, a multi-factorial aetiology has now been proposed with both genetic predisposition and environmental factors (such as infection) associated with disease expression (Walkup *et al.*, 1996). The pathogenesis of TS remains obscure but is considered to be an inherited neuro-developmental disorder resulting in disinhibition of cortico-striatal-thalamic-cortical circuitry (Jankovic., 2001). Although a variety of neurotransmitter abnormalities have been implicated in the pathogenesis of TS, subtle abnormalities of the dopaminergic system remain the most favoured site of pathogenesis (Robertson., 2000; Singer., 2000). As a genetic basis for TS has yet to be uncovered, alternative pathological models are being considered and TS may yet be due to heterogeneous pathologies (Walkup *et al.*, 1996).

Due to the similarities of the symptoms of PANDAS with TS (waxing/waning tics and OCD) it has been suggested that streptococcal infection(s) may also play a role in a subgroup of TS patients (Dale., 2003; Swedo *et al.*, 1998). In comparison of PANDAS with TS, there is a high prevalence of OCD, anxiety, depression and motor disorders in both with recovery of the movement disorder being generally mirrored by recovery of the psychiatric symptoms (Swedo *et al.*, 1998). The finding of similar clinical symptoms to

TS, in the post-streptococcal neurological syndromes, and the fact that they may mimic the relapses and remissions in TS, has lead to speculation that a subgroup of patients with TS may have post-infectious, autoimmune basal ganglia pathogenesis perhaps precipitated by streptococcal infection (Dale., 2003).

Patients with TS have been reported to have significantly higher streptococcal serology (ASOT and anti-DNAse B) compared to control subjects (Cardona *et al.*, 2001; Morshed *et al*, 2001; Muller *et al.*, 2000), which may support a post-streptococcal hypothesis in some patients. Not all studies have, however, have shown this association and has cast doubt on the streptococcal hypothesis of TS (Singer *et al.*, 1998; Singer *et al.*, 1999). Preliminary analysis of antibody reactivity to streptococcal M-proteins has demonstrated higher titers to M12 and M19, but not to M1, M4 and M6 in 25 adult TS patients compared to 25 control subjects (Muller *et al.*, 2001). This may suggest that only certain strains are associated with a subset of TS as has been observed for other streptococcal autoimmune diseases (Bronze *et al.*, 1993; Cunningham., 2003; Muller *et al.*, 2001).

Antibodies reactive against basal ganglia neurons have been reported in TS but studies have not produced consistent findings, perhaps due to variations in detection methodology. Increased antibody binding to putamen rather than caudate neurones has been reported in TS using ELISA and Western immunoblotting, and antibody reactivity against basal ganglia antigens of molecular weights 60, 67 and 83 kDa was proposed (Singer *et al.*, 1998). A second study using Western immunoblotting suggested that ABGA bound to 52, 58, 64 and 88 kDa basal ganglia proteins in TS; although a complex

multivariate analysis was required to establish the association (Wendlandt *et al.*, 2001). Results from another group also suggested that an 83 kDa protein was common in patients with TS and OCD, (Trifiletti *et al.*, 1999; Trifiletti *et al* 2000). However, not all studies have shown similar findings. Two studies using a neuroblastoma cell line as the antigen source rather than human basal ganglia found no discriminating antibody responses in TS (Morshed *et al.*, 2001; Singer *et al.*, 1999). Two other studies using the same cell line did however find increased antibody responses in TS (Laurino *et al.*, 1997; Wendlandt *et al.*, 2001). These discordant results could suggest that only a subgroup of TS patients have a post-streptococcal autoimmune phenotype, implying that the selection and numbers of patients tested for ABGA studies is crucial. Alternatively the differences in methodology IF, ELISA and Western immunoblotting may have a significant impact on results and the choice of antigen may be important (human basal ganglia vs. cell line). Investigations using large cohorts of specimens and detecting the sensitivity and specificity of ABGA using several methods may help to solve this issue.

Volumetric MRI studies in TS cohorts have reported basal ganglia asymmetry or volumetric abnormalities compared to controls (Moriarty *et al.*, 1997; Peterson *et al.*, 1993). This is similar to that found in PANDAS and SC studies. In addition, some MRI studies have also shown that another area of the brain, the corpus callosum was abnormal, with changes usually found in males (Baumgardner *et al.*, 1996; Moriarty *et al.*, 1997). This suggests some patients have predominantly basal ganglia abnormalities whilst others have abnormalities of the corpus callosum, and some even have normal appearances on imaging. This may also support TS as a heterogeneous disorder. A post-mortem study has

reported that there may be basal ganglia involvement in some patients, as differences in striatal dopamine receptor (D1 and D2) binding was found in TS patients (Singer *et al.*, 1991). The studies into dopaminergic abnormalities have been somewhat contradictory (Robertson., 2000; Singer., 2000) showing further evidence that the pathogenesis of TS may be heterogeneous.

1.18 Isolated extrapyramidal syndromes

Although the clinical phenotypes of ABGA-positive individuals include chorea and tics, it is not known if these are the only movement disorders associated with ABGA and streptococcal infection. No post-streptococcal autoimmune studies have yet been reported in dystonia, myoclonus, tremor or parkinsonism or indeed in isolated psychiatric disorders.

1.19 Conclusions

Autoimmunity is an important cause of disease, although the exact mechanisms of disease induction are sometimes unclear, although viruses and bacteria have been implicated. Molecular mimicry is one hypothesis, and the central nervous system disorder, Sydenham's chorea which is post-streptococcal, may be a model of this mechanism. Due to similarity of SC with neuropsychiatric disorders, investigating a common autoantibody in other CNS disorders is warranted.

1.20 Hypothesis

Anti-basal ganglia antibodies are central to the pathogenesis of SC and a significant proportion of patients should be positive. If SC is an autoimmune disease there should be other indicators of immune activation. Anti-basal ganglia antibodies and evidence of streptococcal infection could be important in the pathogenesis of TS as they are proposed to be part of a spectrum of post-infectious neuropsychiatric disorders (PANDAS). Identification of candidate basal ganglia auto-antigens is important to enable us to understand the pathological consequences of ABGA.

2 Methods

2.1 Aims of the methods

- The presence of anti-basal ganglia antibodies (ABGA) was investigated in SC, PANDAS and TS using traditional IF methods against human brain tissue to assess previous published results
- To develop ELISA and Western immunoblotting methods to investigate whether antibodies bind to proteins of similar molecular weight(s)
- Investigate the evidence for streptococcal infection using throat culture when available and surrogate serological markers of infection
- Examine other indicators of autoimmunity, including cytokine levels and oligoclonal bands
- Identify candidate autoantigens using proteomic techniques

2.2 Diagnosis of Sydenham's chorea, PANDAS and Tourette's

Sydenham's chorea

All SC patients were assessed and diagnosed by an associate Professor of Neurology, Dr F Cardoso, who recruited the cases from his outpatient paediatric clinic at the Federal University of Minas Gerais, Brazil. SC was diagnosed if the patient had acute onset of chorea and met the modified Jones criteria for RHF, other causes of chorea having been excluded (Jones., 1945; Special Writing Group of the Committee of Rheumatic Fever., 1992). Acute SC was diagnosed if the patient had chorea lasting less than 2 years regardless of the use of neuroleptics and/or valproic acid. Persistent chorea was

diagnosed if the patient had a diagnosis of acute SC and the chorea lasted 2 or more years regardless of the use of neuroleptics and/or valproic acid. A cardiac assessment, including ECG was performed in all patients with SC. Evidence of active carditis was present in 34/48 of the acute SC patients, 2/24 of the persistent SC patients and all of the rheumatic fever controls.

PANDAS

The diagnosis of PANDAS was made by a paediatric neurologist, Dr. Russell Dale, (Great Ormond Street Hospital) according to clinical criteria (Swedo *et al.*, 1998). Clotted blood samples were taken from patients and controls and centrifuged within one hour to collect serum. All samples were stored at -20°C until required. Throat swabs were also collected for GABHS or other bacterial identification. Each patient was also thoroughly investigated for alternative causes for their disorder which included laboratory investigations for: anti-nuclear antibodies, acanthocytes, amino acid disorders, copper and caeruloplasmin and, where appropriate genetic screening.

Tourette's syndrome

All TS patients were diagnosed by Prof. Mary Robertson using standardised instruments including the National Hospital Interview Schedule (Robertson MM *et al.*, 1996), the Diagnostic Interview Schedule (Robertson *et al.*, 1999) and the Yale Global Tic Severity Rating Scale (Leckman *et al.*, 1989). In order to make a diagnosis of TS, patients had to satisfy DSM-IV-TR (APA 2000) and ICD-10 (WHO 1992) criteria. Thus, patients had to have multiple motor and 1 or more vocal tics, with symptoms lasting longer than 1 year.

2.3 Samples

The control groups for the SC study were community matched healthy controls. None of the SC patients or control groups was or had been on corticosteroids or other immunosuppressive treatment regimes. All patients and controls signed consent for the study, which was authorised by the local ethics committee (Brazil and United Kingdom). Whole (clotted) blood samples from patients with clinically definite acute, persistent SC and controls were taken during the same time period 1999-2000. The blood samples where centrifuged within 1 hour to separate sera which were pipetted into 200µl aliquots and stored at -80°C prior to being couriered to the laboratory on dry ice where they were placed in a -80°C freezer until required (Table 2-1).

Table 2-1 Sydenham's chorea sample demographics (all Brazilian samples)

Group	Number	Mean age years (range)	Sex M/F
Acute SC	34	11.3 (4-20)	8/26
Persistent SC	24	14.4 (11-21)	7/17
Rheumatic Fever	20	16.4 (13-17)	4/16
Normal controls	40	15.3 (12-17)	18/22

2.3.1 Paired cerebrospinal fluid and serum cohort of Sydenham's chorea patients from Brazil

To investigate systemic and intrathecal immunological abnormalities in SC, a second cohort of patients was recruited (Table 2-2). Paired serum and cerebrospinal fluid (CSF)

samples were taken on the same day and snap-frozen immediately after collection in liquid nitrogen and stored at -80 °c until use. All the SC samples were snap-frozen within 10 minutes of collection and stored at -80°c until the samples were couriered on dry ice to the laboratory. No control data for normal CSF cytokine levels had been recorded; 10 patients with a diagnosis of non-inflammatory, neurological diseases were recruited from Great Ormond Street Hospital as controls. These patients were undergoing CSF and serum collection as part of their routine investigations and gave consent for the study, again authorised by a local ethics committee. The final clinical diagnoses of these patients were: Epilepsy (n=2), neurodegenerative disease with dystonia (n=4), benign intracranial hypertension (n=2) and dopamine responsive dystonia (n=2). These samples were taken during the same time-course (2001-2002) and in the same way as the SC patients. The SC and control demographics are presented in table 2-2.

Table 2-2 Sydenham's chorea: Cerebrospinal fluid and control sample demographics

Group	Number	Sex (M/F)	Mean age years (range)
Acute SC	14	2/12	10.2 (4-17)
Persistent SC	4	1/3	13.8 (11-21)
Controls	10	4/6	9.8 (2-16)

2.3.2 Patients with Sydenham's chorea from the United Kingdom

Thirteen children with a diagnosis of acute and four with persistent SC were identified and recruited from the UK during 1999-2003 to assess whether differences in population and access to health care differences influenced streptococcal serology, ABGA positivity

and clinical outcomes. Other causes of chorea were excluded through careful clinical assessment (Neurology, Great Ormond Street Hospital). The patients were diagnosed according to the modified Jones criteria (Jones., 1945; Special Writing Group of the Committee of Rheumatic Fever.,1992). The patients had an acute onset of a movement disorder (chorea) with a clinical history of GABHS infection and/or RHF.
Laboratory testing was performed in each case to exclude other diseases such as SLE, vascular or metabolic disease. MRI was performed where clinically appropriate. In each case the patient and family signed consent (local ethics permission for the study was given) and were assessed and diagnosed by a paediatric neurologist, RCD. Serum was stored at -20°c for testing ABGA and streptococcal serology. In addition a throat swab was obtained and cultured to detect GABHS or other bacteria. CSF was not obtained from any patient.

2.3.3 Control groups used for PANDAS and Tourette's studies in children

For control data, 30 UK children with a diagnosis of development delay were recruited from Great Ormond Street Hospital. To investigate ABGA and streptococcal serology in other streptococcal autoimmune syndromes; 49 UK children with a clinical diagnosis of streptococcal autoimmune diseases (non-neurological disease) were recruited. The streptococcal patients had a clinical diagnosis which was supported by laboratory findings (throat swab, raised ASOT and DNAse B, exclusion of alternative pathogenesis). The clinical phenotypes were: PSGN (n=14 patients), post-streptococcal arthritis (n=15 patients), vasculitis including; Henoch schönlein purpura (n=5 patients), Kawasaki's syndrome (n=10 patients) and rheumatic fever (n=5 patients). This study was authorised

by the local ethics committee and controls were recruited during the same time period as the SC patients (1999-2003). Twenty millilitres of whole (clotted) blood was taken from each patient and control and the serum separated and stored at -80°c until use.

2.3.4 Paediatric autoimmune neuropsychiatric disorders associated with streptococcus

UK patients with a diagnosis of PANDAS that conformed to the clinical diagnostic criteria (Swedo *et al.*, 1998) were recruited. Patients were recruited by Dr. Russell Dale, paediatric neurologist at Great Ormond Street Hospital using the Swedo criteria for a diagnosis of PANDAS (Swedo *et al.*, 1998). Patients with chorea or other neurological diagnosis were excluded also according to the Swedo criteria (Swedo *et al.*, 1998).

Several control groups were also recruited (Tables 2-3 and 2-4). All patients were recruited from the Neurology department at Great Ormond Street Hospital, London. UK. All patients and controls signed consent for this study, which was authorised by the local ethics committee.

Table 2-3 Neurology controls for Tourette's and PANDAS

Dystonic (n=32)	Encephalitis	• Invasive n=5 (HSV, EBV, VZV) • Inflammatory n=4 (Aicardi Goutière and Coats disease) • Rasmussen's encephalitis (n=2) • SSPE (n=1)
	Vascular	• Basal ganglia stroke (n=6) • Vasculitis (n=1)
	Metabolic	• Kernicterus, mitochondrial cytopathy, Batten's disease, Lesch Nyhan, SanFillipo, Glutaric aciduria type 1 (n=1 each)
	Other	• Status dystonicus (n=2), nvCJD, basal ganglia tumour, athetoid cerebral palsy, drug-induced rigidity, juvenile Parkinson's (n=1 each)
Encephalitis with no extrapyramidal features (n=17)	Invasive	• Unidentified organism (n=12), HSV (n=2), mycoplasma (n=1), adenoviral (n=1), neurocysticosis (n=1)
Immune-mediated CNS disorders (n=45)	Demyelinating disorders	• Acute disseminated encephalomyelitis (non-streptococcal) (n=16) • Multiphasic disseminated encephalomyelitis (n=2) • Multiple sclerosis (n=4) • Optic neuritis (n=3)
	'Autoimmune disorders'	• Dancing eyes syndrome (n=9) • Post-infectious ataxia/cerebellitis (n=7) • Chronic infantile neurological cutaneous articular syndrome (CINCA) (n=3)
Other neurology controls (n=6)	-	• Epilepsy (n=3), myasthenia gravis (n=1), metachromatic leukodystrophy (n=1), Bell's palsy (n=1)

Table 2-4 Autoimmune controls without apparent neurological complications (n=70)

Clinical phenotype	Number	Age (range)	Sex (M/F)
Rheumatic fever no chorea	N=16	9.8 (6-15)	5/11
Post-streptococcal glomerulonephritis	N=14	11.8 (9-16)	8/6
Post-streptococcal vasculitis	N=8	13.4 (10-17)	3/5
Erythema nodosum	N=4	12.4 (9-15)	2/2
Post-streptococcal arthritis	N=15	16.2 (11-17)	7/8
Post-streptococcal uveitis	N=4	9.9 (8-10)	2/2
Juvenile idiopathic arthritis	N=4	14.3 (12-16)	3/1
Henöch Schonlein purpura	N=3	11.9 (10-13)	2/1
Systemic lupus erythematosus, Kawasaki's disease, polyarteritis nodosa and Cogan's syndrome	N=2 each	15.6 (9-17)	2/6

2.3.5 Patients with Tourette's syndrome

I decided to investigate the hypothesis that some patients with Tourette's syndrome may fulfill the criteria for PANDAS. Children and adults with TS were recruited to investigate differences in evidence for a PANDAS-like pathogenesis in different TS age groups. A large cohort of patients was screened to assess the proportion with evidence of streptococcal infection, by measuring ASOT compared to controls. ABGA was also assessed by ELISA, Western immunoblotting and IF. Ethics permission was obtained for the cross-sectional study from the National Hospital for Neurology and Neurosurgery and the Institute of Neurology's local ethics committee. The TS patient and control patient demographics are presented in Table 2-5.

Seven TS patients were also recruited for a follow-up study after primary testing. A whole blood sample was taken 3 months after the initial blood sample and the serum was retested for ABGA and streptococcal serology. This was because the intensity of tics and neuropsychiatric symptoms waxes and wanes in TS as it does in PANDAS (Swedo *et al.*, 1998). It was hypothesised that the exacerbations in TS were related to the presence or absence of streptococcal infection(s) which could cause an increase or production of ABGA leading to neuronal dysfunction with worsening of TS symptoms.

Table 2-5 Tourette's syndrome patient and control demographics

Group	Number	Mean age years (range)	Sex (M/F)
Child Tourette's Syndrome	56	12.8 (8-17)	40/16
Adult Tourette's Syndrome	44	37.8 (18-61)	32/12
Child streptococcal infection controls	50	9.8 (2-15)	25/15
Child neurological controls	50	7.6 (0.5-18)	24/26
Adult healthy controls	50	35.6 (19-57)	25/25
Adult neurological controls	50	41.1 (19-70)	30/20

A number of control groups (adults and children) were recruited to assess sensitivity and specificity of ABGA and streptococcal serology in the TS study. Recruitment of controls occurred during the same time period and samples were stored in the same manner. The demographics and clinical definitions of each control group are presented in Tables 2-6, 2-7, 2-8 and 2-9.

Table 2-6 Neurological control groups (children) for Tourette and PANDAS

Group	Diagnosis	Number	Mean age in years (range)	Sex (M/F)
Dystonic	Inflammatory	12	10.9 (3-13)	5/7
	Vascular	6	11.2 (5-12)	2/4
	Metabolic	6	8.9 (2-11)	4/2
	Genetic	2	7.0 (6-8)	2/0
Encephalitis	HSV, VZV, EBV and unknown 'viral'	20	12.2 (5-13)	12/8
Other	Variant CJD, basal ganglia tumour, cerebral palsy, juvenile Parkinson's disease	1 each	10.5 (7-11)	2/2

Table 2-7 Streptococcal infections (childhood) controls for the PANDAS and SC Study

Group	Number	Mean age in years (range)	Sex (M/F)
GABHS pharyngitis	45	13.8 (3-16)	20/25
GABHS skin infections	5	9.9 (2-13)	3/2

Table 2-8 Neurological disease controls (adults) for the Tourette's study

Group	Number	Mean age in years (range)	Sex (M/F)
Multiple Sclerosis	12	38 (30-52)	2/10
Encephalitis	20	28.9 (19-36)	11/9
Dementia	7	63.6 (55-74)	4/3
Paraneoplastic syndrome	3	60 (59-63)	2/1
Autoimmune Neuropathy	3	32.5 (29-34)	1/2
Cerebellitis	3	42 (40-43)	1/2
Other	2	41 (40-42)	1/1

Table 2-9 Healthy adults recruited from staff in the laboratory staff and the Great Ormond Street Hospital for the Tourette's study

Group	Number	Mean age in years (range)	Sex (M/F)
Healthy adults	50	35.6 (19-57)	25/25

2.3.6 Adult onset movement disorders

Adult patients with an acute onset movement disorder were recruited from the movement disorder clinics at the National Hospital of Neurology and Neurosurgery, London. All patients were being routinely investigated for the cause of their movement disorder and ethical permission was obtained to investigate the presence of ABGA and streptococcal serology. The patient demographics are presented in table 2-10.

Table 2-10 Adult movement disorders: sample demographics

Diagnosis	Number	Sex (M/F)	Age years (range years)
Primary blepharospasm	7	2/5	38.7 (25-52)
DYT1 positive dystonia	10	4/6	41 (35-45)
Idiopathic dystonia	20	10/10	34 (26-40)
Huntington's disease	3	2/1	45.6 (35-50)
Tourettism	5	5/0	39.9 (26-45)

To answer the question as to whether ABGA are associated with an autoimmune basal ganglia syndrome such as the proposed pathogenesis of SC, adults with a range of movement disorders were also investigated. Patients with genetic disorders such as Huntington's disease and DYT (gene) positive dystonia were studied to see if ABGA might be epiphenomena to secondary basal ganglia pathogenesis. Primary blepharospasm can be associated with autoimmunity in 26% of patients (Jankovic *et al.*, 1993). The presence of an inflammatory pathogenesis in these patients may have led to the production of autoantibodies against brain tissue such as the basal ganglia so, was also investigated. Idiopathic dystonia and adult-onset "Tourettism" were also investigated as the range of movement disorders associated with streptococcal infection and ABGA may be wider than previously thought.

2.4 Basic laboratory testing of samples: immunoglobulin measurements

Serum IgG levels were measured by routine rate nephelometry in the SC patients to assess whether an increase in polyclonal IgG as a consequence of past streptococcal infection may lead to false positive binding to the basal ganglia tissue and lead to aberrant results in the ABGA ELISA method. Samples were also tested for ASOT and anti-DNAse B.

2.5 Indirect immunofluorescence

2.5.1 Commercial sections

Sagittal brain sections from rat were purchased from Novagen, Germany. Monkey sections were purchased from The Binding Site, UK. Control tissue sections containing monkey myocardium (The Binding Site FS215), cerebellum, cerebrum and mouse stomach (The Binding Site FS224.A) and mouse liver, kidney and stomach (The Binding Site FS313) were purchased commercially. Sections were formalin fixed and prepared for IF using standard techniques.

Basic method

- Sera was diluted 1/50 and 1/500 in 0.9% saline and incubated for 30 minutes
- Sections were washed in 0.9% saline for 20 minutes
- Sections were incubated with anti-human IgG conjugated with FITC (Dako) diluted according to manufacturers instructions
- Sections were washed in 0.9% saline for 20 minutes
- Sections were mounted in permanent mounting fluid (Dako)
- Sections were screened using a fluorescence microscope (Leitz) the same day

2.5.2 Human sections

A tissue block containing human dissected caudate and putamen from the Queen Square Neurological Diseases Brain Bank, University College London, was snap-frozen in iso-pentane (Fisher Scientific P/1030) and frozen carbon dioxide pellets. A commercial preparation of tissue embedding medium, tissue-tek OCT (Sakura Finetek) was used to create a tissue block for microtome sectioning. The block was held in place with tissue-tek and placed in the freezing iso-pentane to form a frozen tissue block by attaching the frozen tissue to a metal chuck which was used to cut sections on a freezing microtome. The chuck was kept at -80°C until use and then transferred to the microtome which was kept at -20°C to facilitate cutting. The chuck was left for 30 mins to equilibrate and then excess tissue tek medium was trimmed from the block using the microtome to reveal the longitudinal or transverse section of caudate/putamen or globus pallidus. Briefly, 10 µm thick frozen sections were cut from the block and a glass slide was gently placed onto the section which was attached to the surface of the slide. The slides were labelled and were immediately stored at -80°c.

2.6 Basic method of indirect immunofluorescence to detect anti-basal ganglia antibodies

Basic method

- Tissue sections were allowed to defrost at room temperature for 30 mins before use.
- The sections were blocked with 10% normal rabbit serum (Sigma R9133) in saline (Sigma P4417), (blocks exposed proteins thereby reducing false positive staining) for 30 mins in a damp chamber at room temperature to prevent drying.
- The sections were gently washed and placed in a slide tray containing the same PBS wash solution to soak for 20 mins to remove unbound blocking solution.
- The slides were removed from the bath and excess fluid was wiped from around the edges of the sections to prevent additional dilutions of sample and detector reagents.
- During the blocking step, patient and control serum samples were diluted with PBS containing 0.1% rabbit serum. The 1/50, 1/500 diluted samples (200μL) were carefully pipetted onto each slide and allowed to incubate for 30 mins in a damp chamber
- The serum was gently washed off with PBS and the slide washed with PBS to remove excess sera. After 20 mins each slide was removed from the bath and excess fluid carefully wiped from around the edges of the section, ensuring the section did not dry out.

- Rabbit anti-human IgG secondary antibody conjugated with fluorescein isothiocynate (FITC), (Dako F0315) was diluted 1/30 with PBS/0.1% rabbit serum and applied to each slide to detect antibody binding.
- The sections were washed as previously described. Sections were mounted (see appendix) and kept at +4°C in the dark until screened (the same day) using a fluorescence microscope (Leitz, UK).
- Sections were assessed for apple-green staining of neurons (Husby *et al.*, 1976) and photographs of typical findings from controls and SC patients were taken using an attached camera (Olympus, UK).

2.6.1 Identification of individual cell types

Differentiation of the cell types on the IF staining patterns such as glial cell population (neuronal supporting cells) were identified by testing the same tissue sections with a diluted (1/500) monoclonal antibody specific for glial fibrillary acid protein (GFAP), (Sternberg Monoclonals Inc). The neurons were identified (stained) by using a diluted monoclonal antibody (1/500) specific for neuron-specific enolase (NSE), (Santa Cruz biotechnology sc-21737). The staining of neurones and accessory cells was carried out by comparing the pattern of fluorescence (FITC secondary detector). The staining pattern obtained using the GFAP and NSE monoclonal antibody was compared to that of patient serum (IgG) from SC, and PANDAS.

2.6.2 Comparison with previous published immunofluorescence results

The IF method previous published (Husby *et al.*, 1976; Kotby *et al.*, 1998) was applied to the detection of ABGA in SC patients and controls by testing samples from acute SC and normal controls undiluted or diluted 1/2 on basal ganglia sections. The sections were then assessed by counting the number of stained neurones and grading the intensity of staining in both SC and controls as previously proposed (Husby *et al.*, 1976; Kotby *et al.*, 1998). To improve on this IF method (background and false positive staining) the standard dilution of serum was changed to improve assessment of positivity by enabling the differences between positive and negative to be clear through the presence or absence of neuronal staining. Serial dilutions of SC and control samples were prepared (1/2, 1/10, 1/20 and 1/100) in PBS. The optimum serum dilution for testing SC samples by IF was assessed as the serum dilution which resulted in the ideal signal to noise ratio of stained neurons with low background and the absence of non-specific binding or background auto-fluorescence. Positive staining was interpreted as apple-green staining of the basal ganglia neurons with sparing of accessory glial cells.

2.7 Systemic (non-brain) autoantibodies

Rat tissue sections of liver, kidney and stomach (RLS) were used for antibody binding with IF to detect systemic autoantibodies. These included anti-nuclear, smooth muscle, parietal cell, liver-kidney microsomes, reticulin, mitochondrial and ribosomal antibodies. Any positive reactivity or titre was recorded for each patient and control sample tested.

2.7.1 Indirect Immunofluorescence against rat tissue

Snap-frozen RLS tissue sections (The Binding Site FS313) were used

Basic method

- SC and controls which were diluted 1/10 and 1/25, pipetted on to each section and incubated for 30 mins at room temperature.
- Diluted serum was washed off with PBS and sections were placed in a bath containing PBS for 20 minutes.
- Rabbit anti-human IgG conjugated with FITC (Dako P0315) was diluted 1/20 and carefully pipetted onto each slide and incubated for 30 mins.
- Sections were washed in PBS and left in fresh PBS for a further 20 mins.
- Sections were mounted and specific binding was assessed using a fluorescence microscope (Letiz) the same day.

2.8 Human brain tissue for ELISA and Western blotting

Introduction

Human brain tissue was used rather than rat or rabbit brain because the original reports of ABGA were all carried out using human brain. This was available for use. Rat brain was also being investigated as a source of antigen in PANDAS sera by Dr Russell Dale in the same laboratory, therefore this was not duplicated.

Whole human striatum, containing caudate, putamen and globus pallidus, was obtained from the Queen Square Neurological Diseases Brain Bank, University College London, from a cadaver that had no clinical or histological evidence of neurological disease. This was authorised by the local ethics committee at the National Hospital for Neurology and Neurosurgery, Queen Square, London. Human cerebellum, cerebral cortex, myelin and spinal cord were also obtained from the Queen Square Neurological Diseases Brain Bank and used as control tissue for autoantibody determination in patients and controls. The preparation of tissue to provide antigen homogenates for antibody assays was carried out using the same methods as those for the basal ganglia homogenates. The tissue had been dissected from the brain by a qualified brain pathologist and flash-frozen 24 hours after death. The dissected tissue was stored at -80°C prior to further preparation for anti-basal ganglia antibody assays.

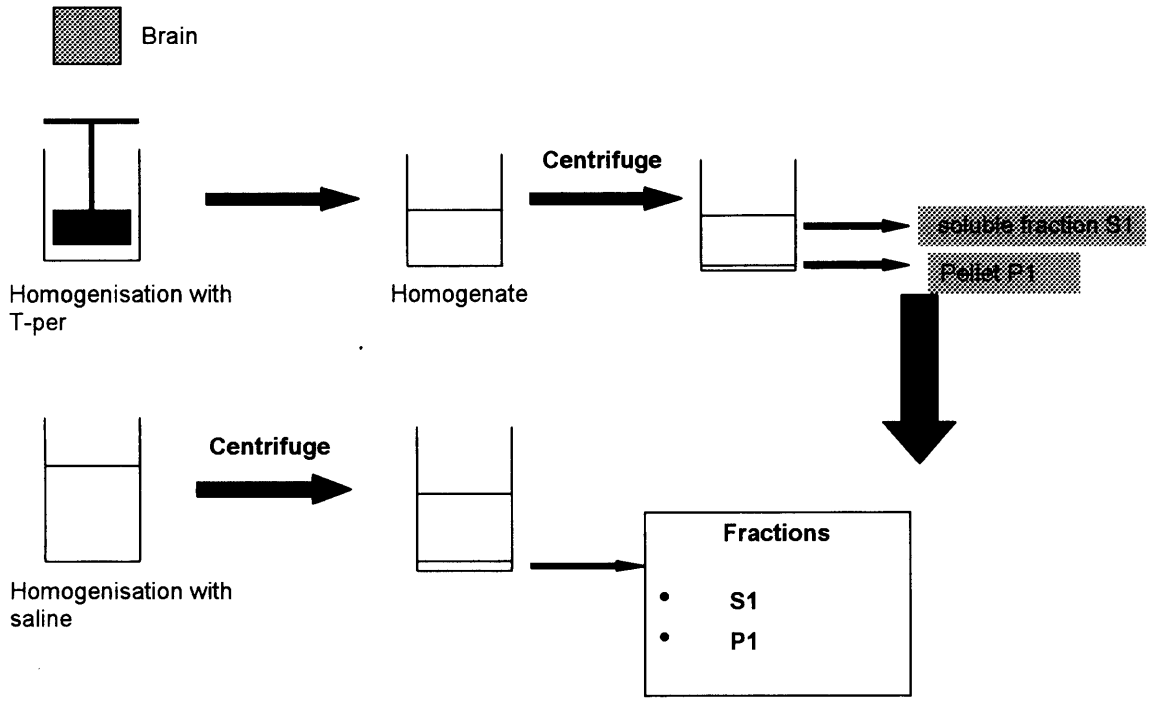
2.9 Homogenisation of brain tissue with saline

A block of brain tissue was defrosted at room temperature

Basic method

- Tissue was weighed and cut into 10mm pieces using a sterile scalpel
- The tissue pieces were placed in a glass homogenizer tube (Fisher scientific) which had been stored on water ice for 30 mins to cool
- Add protease inhibitor cocktail was immediately added to the tube (Sigma P-8340) in a concentration of 500µL per 2g of tissue
- The tissue was mechanically homogenised with a small volume of sterile saline 0.5mL/g until all visible aggregates of tissue had disappeared.
- The resulting homogenate was pipetted into sterile tubes and centrifuged at 10,000 rpm for 30 mins to remove large particles and insoluble material.
- The resulting supernatant was carefully pipetted off and stored at -80 °c until required (Soluble S1 protein fraction), Figure 2-1.
- The insoluble cell debris at the bottom of the centrifuged tube was also stored, (fraction pellet P1).

Figure 2-1 flow diagram showing protein preparation protocol



Centrifuge 10,000 rpm for 30 minutes, fractions stored at -80°c.

2.10 Homogenisation with commercial detergents

Homogenisation of striatum was also carried out using a commercial non-ionic detergent cocktail, tissue protein extraction reagent, T-per (Perbio 78510), (appendix), which was added to the homogeniser tube instead of saline. The addition of T-per is reported to increase the efficiency of protein recovery from tissue by gently breaking down cell membranes and increasing the release of proteins into the supernatant (see appendix). However, T-per is probably no better than the addition of non-ionic detergents such as Triton X or CHAPS in improving solubilisation but is reproducible and free of contaminants.

The T-per reagent was compatible with most assays, including Western Immunoblotting and ELISA and was capable of being dialysed. Reactivity to commercial antibodies was assessed using T-per and saline fractions to investigate its use.The method for T-per protein homogenisation was as the same as that for saline. The resulting supernatant and pellets were pipetted into fresh tubes. The fractions were stored at -80°C until used.

2.10.1 Demyelinated fractions

The antibodies against basal ganglia are thought to bind to neurones (Husby *et al*, 1976). However, it had not been shown whether this antibody binding was specific to a particular neuron type, or indeed was directed against the myelin sheath of neurones. As antibody responses against myelin sheath components have been shown previously in a number of neurological diseases, including MS and encephalitis, it was essential to investigate whether antibodies from patients with SC and PANDAS reacted against the

myelin components of neurones or neuronal proteins. The myelin containing fraction of the prepared supernatants was separated to allow antibody activity against myelin and neuronal proteins to be assessed separately.

Myelin was removed as followed:

- 2 mL aliquot of basal ganglia homogenate was pipetted into a glass tube and 500μL of the solvent, di-iso-propyl-ether (BDH 28268) was added.
- The tube was sealed with a glass stopper, mixed thoroughly and centrifuged (Whatman Force 712) at 10,000 rpm for 15 mins in a sealed centrifuge bucket.
- The protein fraction was carefully pipetted from the protein/lipid interface using a sterile spinal needle and syringe.
- The protein fraction was transferred into another tube and centrifuged again to remove any remaining lipid which had been pipetted from the surface.
- The lipid fraction was then pipetted from the tube avoiding contamination with the solvent.
- The protein concentrations were determined and the fractions stored at -80°c.

2.10.2 Antibody reactivity in T-per and saline produced fractions

Commercial antibodies against neurone-specific enolase (Autogen Bioclear), pyruvate kinase and Aldolase C (AbCam) were diluted 1/5000 and tested against Western blots of saline and T-per produced fractions to ascertain if T-per might affect antigen reactivity.

2.10.3 Protein assay

All the homogenised tissue was assayed for protein concentration by using a commercially available kit based on the Lowry method (Biorad 500-0114). Standards

containing a known concentration of protein were prepared (0, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/mL) along with a reagent blank (distilled water)

Basic method

- Standards and controls and sample were pipetted in duplicate into a 96 well microtitre plate (BDH 402200432)
- A series of doubling dilutions of each homogenate was prepared (1/2-1/64). 10µL of neat and diluted homogenates was pipetted in duplicate into the microtitre plate
- 25µL of reagent A was added followed by 200µL of reagent B. The plate was left for 15 mins to develop at room temperature
- Absorbances were read on a plate reader (Wallac) at 790nm with a reference filter of 492nm
- The mean absorbance for duplicate wells was calculated for each standard and homogenate dilution
- The standard curve was plotted using excel software (Microsoft)
- The protein concentration for the neat homogenate and dilutions was extrapolated from the curve and recorded. Dilutions were used when the neat protein concentration was above the highest protein standard

2.11 Anti-basal ganglia antibodies: enzyme-linked immunoabsorbant method

2.12 Method development

An enzyme-linked immunoabsorbant assay (ELISA) to detect ABGA was designed to allow the screening of large numbers of serum samples simultaneously. The results from the ELISA method also enabled statistical analysis of data of controls and post-streptococcal neurological disease patients. The differences in the ELISA results obtained using antigen derived from: 1). Soluble S1 fraction of the whole basal ganglia homogenate; 2) The S1 fraction treated to remove lipid; 3) The insoluble P1 fraction. The ELISA assay was developed from 16 ABGA IF positive stained samples. Normal and neurological disease controls were used to develop a cut-off value of absorbance (the upper limit of normal) to assess the percentage of patients and controls with raised ABGA ELISA results.

2.12.1 Antigen dilutions and method development

Initially, aliquots of each basal ganglia antigen were defrosted and diluted with 0.5M carbonate buffer to produce aliquots containing: 5µg/mL, 2.5µg/mL, 1.25µg/mL, 1.0µg/mL and 0.5µg/mL of total protein.

Basic method

- Microtitre plates (BDH maxisorb 402200432) were coated with 100µL of each antigen using 0.05M carbonate buffer (see appendix) as a coating medium.
- Plates were covered with Clingfilm and left overnight (maximum of 14 hours) at +4°C.
- The plates were allowed to come to room temperature and washed four times with 0.2% bovine serum albumin (BSA), (Sigma A7906) diluted in PBS containing 0.05% Tween 20 (Sigma P7949) using a commercial plate washer (Biorad).
- Plates were blocked with 2% BSA/PBS and incubated for 1 hour at room temperature with gentle shaking.
- The microtitre plates were washed 4 times with 0.2% BSA/PBS, 0.05% Tween 20 (Sigma P7949) to remove excess blocking reagent.
- Serum samples from the controls and acute SC patients (IF positive) were initially diluted 1/100 in 0.2% BSA/PBS with 100µL pipetted into each well in duplicate.
- Two wells were used as a reagent blank by pipetting 100µL of 0.2% BSA/PBS without serum into duplicate wells.
- The plates were incubated at room temperature for 1 hour with gentle shaking on a plate shaker (Denley, UK) and washed as previously described.

- Freshly prepared 1/1000 diluted anti-human IgG secondary antibody conjugated with horseradish peroxidase (Dako P-0406) was added to each well and incubated for 1 hour at room temperature.
- The plates were again washed as previously described. Colour reagent *O*-phenylenediamine (see appendix) was added to each well and the colour reaction was allowed to develop in the dark for 15 mins.
- The colour change was stopped with the addition of 1M HCL (BDH) to each well and the absorbances were read on a plate reader at 492 nm wavelength with a reference filter of 405 nm (Wallac multi-label counter).

The mean ELISA absorbance for the duplicate wells of blanks, control and IF-positive SC samples was calculated and the results produced from testing different antigen concentrations were compared. The antigen concentrations which gave the best signal to noise ratio (blank wells <0.1 absorbance and normal controls <0.5 absorbance) were used to assess the appropriate antigens for use in the ELISA. The optimum dilutions for testing serum samples and the detector secondary antibodies were analysed by setting up a chequerboard of dilutions against each of the antigen concentrations. The samples tested in each case were the same samples with a moderately high absorbance SC patient (IF positive) identified from the first experiments and a normal control which was IF negative.

2.12.2 Optimum serum dilutions

To investigate the optimum serum dilution for ELISA a chequerboard of dilutions was tested.

Method

- 16 SC IF positive samples were diluted as follows: 1/50, 1/150, 1/300, 1/500 and 1/1000.
- Samples were tested against 1µg/mL of the soluble S1 fraction of basal ganglia
- The mean absorbance was calculated for each of the duplicate wells and plotted against the serum dilutions.

The results were compared against the same samples with different concentrations of the detecting secondary HRP-conjugated antibody.

2.12.3 Optimum dilution of secondary antibody

The anti-Human IgG, HRP conjugated secondary antibody was diluted according to manufacturers instructions 1/2000.

2.12.4 ELISA using protein and myelin containing fractions

The difference in the ELISA results using either lipid extracted antigen preparations or lipid containing homogenates were assessed by running 16 IF positive patients and 16 normal controls against the same protein concentrations of whole basal ganglia homogenate, or the homogenate treated to remove the lipid fraction. The plates were set up at the same time, using the same aliquot of diluted samples. All samples were tested in duplicate with blank reagent wells on each plate. The absorbances were calculated and

the mean results of the negative controls and acute SC patients were compared to assess differences in absorbances between antigens containing purified protein or crude homogenates with lipid. This was repeated using the insoluble fraction.

2.12.5 Basal ganglia antibodies: cut-off value for upper limit of normal absorbances

A statistical cut-off value for assessing the upper limit of normal for absorbances was initially calculated by testing 30 acute SC patients and 30 normal controls against a basal ganglia ELISA with the optimum antigen concentration, serum sample and secondary antibody dilutions. To produce the cut-off value, the mean ELISA absorbance and SD of all the normal controls was calculated and 2 standard deviations (of the control data) added. All patients with SC from the Brazilian cohort and the patients with SC from the UK were analysed and mean absorbances recorded and compared to the cut-off value so that the percentage of patient and controls with raised ELISA results could be assessed.

2.13 Anti-basal ganglia antibodies: Western immunoblotting

Introduction

Lithium dodecyl sulphate (LDS) sample buffer (Invitrogen NP0002) was used in place of the typical sodium dodecyl sulphate (SDS) detergent. Whilst SDS is the usual method, LDS was available to use in the laboratory and is the routine method in use by the Neuroimmunology laboratory, UCL NHS trust for detecting anti-neuronal antibodies by Western blot for the diagnosis of paraneoplastic disease. There were no funds left from my grants to investigate the difference in results obtained from LDS or SDS which is of course a criticism of this Study.

To prevent disulfide bonds forming between cysteine residues a reducing reagent, dithiothreitol (DTT), (Sigma D9163) was added to all the sample preparations. The thiol reagent had the effect of increasing band sharpness by aiding protein separation and reproducibility of the polyacrylamide gel electrophoresis (PAGE).

Conclusion

Therefore in conclusion Western blotting was carried out using the Invitrogen mini-gel, Nu-PAGE system with LDS buffer under reducing conditions and blotting using a semi-dry electro-transfer onto supported nitrocellulose membranes. This was the most studied and expensive part of the thesis and the UCL central research grant paid for it.

2.14 Basic poly-acrylamide gel electrophoresis method

- The basal ganglia homogenate was prepared by adding 250μL of LDS (x 4 concentrate) to 500μL of basal ganglia homogenate.
- In addition 100μL of 0.5M concentration DTT (Sigma) was added to provide a reducing agent of a final concentration of 0.05M. Finally 150μL of ultra-pure, double-distilled water was added and the tube was vortexed to mix sample components thoroughly.
- The tube was then placed into a pre-heated water bath (Grant, UK) at 65°C for 15 mins to denature the proteins. The homogenates were allowed to cool and vortexed again to ensure thorough mixing of proteins. This was used as a stock solution to optimise the protein concentration.

A pre-stained molecular weight marker (Invitrogen, LC2626) was run in one well of each gel. A commercial buffer tank, electrode and power pack (Invitrogen) was used to run the gels with MES running buffer (Invitrogen NP00002) which gave the optimum separation of small and medium size proteins. The gels were run for 39 mins at room temperature using the following electrophoretic conditions: 200V, 120mA and 25W. Once the electrophoresis was finished, a gel was either transferred into fixative solution (see appendix) for staining with Commassie Brilliant Blue to visualise the separated proteins or was electro-blotted, to transfer basal ganglia proteins onto a nitrocellulose membrane (Sartorius WPHY320F2). Nitrocellulose provided a permanent support for investigating protein separation and allowed probing with antibodies. This enabled serum

from patients and controls to be tested against a nitrocellulose strip containing a range of separated basal ganglia proteins (2-200 kDa).

2.14.1 Basic method of electro-blotting of proteins onto nitrocellulose

Transfer of proteins was carried out using electro-blotting, which increased the efficiency of protein transfer on to the nitrocellulose membrane. The procedure uses a semi-dry system and a blotting cassette (Invitrogen E19051). A commercial protein transfer buffer (Invitrogen NP0006), (see appendix) and 20% methanol were used to increase the efficiency of protein transfer.

Basic method

- An electrophoresed gel was removed from its plastic supporting cassette and the gel overlaid with a nitrocellulose membrane which had been pre-soaked in transfer buffer.
- The gel and nitrocellulose was placed on a plastic blotting cassette on top of 2 sponges which had been soaked in transfer buffer.
- A piece of blotting paper soaked in buffer was placed on top of the sponges and the gel placed on top.
- Two sheets of transfer buffer soaked filter paper were placed on top of the nitrocellulose membrane.
- The 'sandwich' was finished by adding 2 transfer buffer soaked sponges and the top of blotting cassette.
- The blotting cassette was placed in the electrophoresis tank (Invitrogen E10001).

- The outer core of the tank contained distilled water to provide heat exchange as the electrophoretic transfer produces heat which could interfere with efficient protein transfer.
- The proteins were electro-blotted onto the nitrocellulose membrane for 2 hours using the following electrophoretic conditions: 25V, 160mA and 17W at room temperature.

The blotted nitrocellulose membrane was carefully removed from the gel and placed into a blocking solution containing 2% skimmed milk powder diluted in 0.9% saline with gentle rocking for 2 hours. This reduces background staining through blocking unbound protein sites with milk proteins. The blocking solution was rinsed off with 0.9% saline.

2.14.2 Basic method of testing samples against Western blots

- The nitrocellulose membrane was placed into a ten well plastic incubation manifold (Amersham Pharmacia 80-6087-98) which allows up to 9 samples to be tested against a lane of separated proteins.
- The incubation manifold was left covered in Clingfilm with gentle rocking overnight at +4°C. The diluted serum in each well was discarded
- The nitrocellulose was washed with water 10 times and then washed with 0.2% milk in saline containing 0.05% Tween 20 (Sigma P-1379) 10 times with 10 min intervals between washes to remove unbound antibody.
- Anti-Human IgG conjugated with HRP (Dako, P-0406) was diluted 1/1000 in 50ml 0.2% milk in saline and left with gentle rocking for 2 hours at room temperature.

- The nitrocellulose was washed as previously described.
- Identification of antibody reactivity was carried out using 4-chloro-1-napthol (Sigma chemical C8890) and hydrogen peroxide as the enzyme substrate.
- The substrate was added to the nitrocellulose membrane and left to develop for 20 minutes at room temperature with gentle rocking.
- The molecular weights of any resulting bands were recorded and the nitrocellulose was scanned using a scanning densitometer (Biorad GS 690) for future analysis.

2.14.3 Optimising Western immunoblotting method

The optimum antigen concentration was assessed by testing basal ganglia homogenates diluted (1/2, 1/3, 1/4, 1/8, 1/10, 1/12, 1/15, 1/20 and 1/40) of whole, de-lipidated and insoluble basal ganglia homogenates S1 and P1 fractions (0.1-100µg/mL).

- 16µL of the prepared dilutions were pipetted into 4-12% Bis-Tris PAGE gel (Invitrogen) with a 10 well configuration and electrophoresed.
- One gel was stained with Commassie Brilliant blue to assess protein separation.
- Duplicate gels were electroblotted onto nitrocellulose and used to test 10 ABGA IF positive samples and 10 neurological controls (1/300) against the antigen dilutions.
- The optimum signal to noise ratio was the antigen concentration which produced bands in SC samples without homogenous staining of the background.

Once protein concentration had been optimised a gel with a 2-D configuration (1 well for MW marker, 1 large well for homogenate) was set up.

- IF positive SC patients and control samples were diluted in 0.2% milk in saline (1/00, 1/200, 1/300, 1/400, 1,500 and 1/1000) and tested using an incubation manifold which allowed 10 dilutions to be tested against one coated membrane.
- The reproducibility of results and stability of the antigens was also determined.
- Five IF positive samples from patients with acute SC were tested against 5 separate Western blots made using the same antigen aliquot. The molecular weight of any reactivity (bands) was recorded and was used to assess reproducibility of band patterns.

2.14.4 Antigen stability for Western blotting

The stability of the antigen source was determined by producing 2 aliquots of basal ganglia homogenates and storing one at +4°c and another at -80°C. Four Western blots were made each week using both aliquots and the same 4 positive (SC) and 4 negative samples tested each time. The molecular weights of reactivity were recorded as was the condition of the results (high background and loss/increase in the number of bands). This information was used to ascertain the optimum storage conditions of the prepared antigen samples and degradation of proteins over time.

2.15 Polyacrylamide gel staining methods

To assess protein separation, band intensity and aid protein identification, PAGE gels were stained to reveal the protein separated into bands using either colloidal blue stain or the more sensitive silver stain.

2.15.1 Colloidal blue stain

The PAGE gel was fixed (appendix) for 10 minutes at room temperature with gentle shaking. The colloidal blue stain was obtained from a commercial source (Invitrogen LC6025), (see appendix). The gels were left in staining solution overnight (8 hours) and the background cleared by several changes of distilled water for at least 7 hours at room temperature.

2.15.2 Silver staining

Silver staining of PAGE gels enabled the staining of proteins down to a nanogram range of concentration, which was up to 100 times more sensitive than blue colloidal staining. A commercial kit was used in all silver staining (Amersham-Pharmacia 17-1150-01) of PAGE gels.

Basic method

- A mini-gel or large SDS gel (Amersham Pharmacia 80-6002-21) was fixed (see appendix) for 30 mins.
- A sensitising solution was added to the gel consisting of 75mL ethanol, 10mL sodium thiosulphate, 17g sodium acetate and 165mL of distilled water and left for 30 minutes.

- After washing in water the silver solution, containing 25mL silver nitrate and 125mL of distilled water, was added with 100µL of formaldehyde (37% w/v) added at the last minute.
- The gel was left for 20 minutes before washing in water and the addition of developing solution which consisted of 250mL distilled water, 6.3g sodium carbonate and 50µL of formaldehyde (37% w/v).
- After 5 mins or once protein 'spots' were visible with clear background, a stop solution containing 250mL distilled water and 3.7g of sodium EDTA was added and left for 10 minutes.

2.15.3 Gel storage

Stained gels for analysis of bands by proteomics and mass spectroscopy were stored in distilled water at 4°C for a maximum of 1 week. For permanent storage stained gels were dried. The PAGE mini gels were dried using a commercial gel-drying system (Invitrogen N12387). A gel was soaked in gel-dry solution for a maximum of 5 minutes and then sandwiched between cellophane which had also been soaked for 10 minutes in gel-dry solution. The gels were left at room temperature away from sunlight for 48 hours to dry. Large SDS gels were soaked for 20 mins in a preserving solution (see appendix). Permanent storage of gels was carried out by wrapping the gel in one sheet of cellophane (Amersham Pharmacia 80-6117-81) and leaving to dry for 48 hours at room temperature away from direct sunlight.

2.15.4 Gel imaging

Where possible pictures were taken using a Sony, Cybershot DSC-V1 5 mega pixel camera, no software manipulation of images was carried out. A Biorad, scanning densitometer was used for older images; no software manipulation of images was carried out.

2.16 Anti-neuronal antibodies against other brain areas

2.16.1 Indirect Immunofluorescence

Monkey cerebellum and cerebrum sections were purchased commercially (The Binding Site, FS224.A). Acute SC patients with positive ABGA were tested at 1/25 and 1/50 against the section to ascertain whether ABGA were specific to striatum.

2.17 Streptococcal serology

Evidence of prior streptococcal exposure was made using 2 independent serological tests (ASOT and DNAse B) when possible. The normal ranges were in accordance with WHO guidelines. A reduction in titre during recovery was necessary to determine the significance of raised streptococcal serology in addition to a clinical determination of likely infections such as pharyngitis or skin infection. Serum ASOT and DNAse B were measured in the controls, SC and PANDAS cohorts to assess whether there were any differences in streptococcal serology suggestive of differences in disease latency or presence of persistent infection.

2.17.1 Anti-streptolysin-O titres (ASOT)

A commercial nephelometry kit was used to measure anti-streptolysin O titre (ASOT), (The Binding Site ZK189.L.R).

Basic method

- Serum or kit controls (normal and raised) were diluted 1/40 and 30µL was pipetted into a cuvette containing a stirring bar.
- Using an electronic pipette 400µL of ASO buffer and 40µL of reaction reagent was pipetted into the cuvette.
- After 2 mins the ASOT result was produced as IU/mL (International units per millilitre).

The upper limit of normal was 200 IU/mL which is in accordance to WHO guidelines (Spaun *et al.*, 1961).

2.17.2 Anti-DNAse B titre

A Dade Behring BN 11 nephelometer and reagent kit (Behring) was used to measure Anti-DNAse B using commercial antiserum and reagents. The upper limit of normal for Anti-DNAse B antibody results was 300 IU/ml (in house laboratory cut-off). This assay was not available for the TS study. This assay was kindly carried out by the Microbiology Department at Great Ormond Street Hospital.

2.18 Cytokine assays

Two Th1 cytokines (interferon-gamma and interleukin-12), (and two Th2 cytokines (interleukin-4 and interleukin-10) were measured using commercial ELISA (Amersham Pharmacia, RPN2783 IL-4, RPN2785 IL-10, RPN2787 δ-INF, RPN2752 IL-12). The method used an antibody specific for the individual cytokine coated onto a microtitre plate.

Basic method

- Test samples standards of known cytokine concentrations and controls were pipetted into duplicate wells and allowed to incubate at room temperature for 1 hour.
- The excess serum or CSF was washed off with several changes of buffer and a biotinylated antibody was then added which binds to the specific cytokine from the incubated sample bound to the microtitre plate.
- An amplification reagent was then added to increase sensitivity and once washed thoroughly to remove unbound antibody
- enzyme substrate TMB was added and the plate allowed to develop for 15 mins.

- The colour reaction was stopped with sulphuric acid and the absorbances of each well read on a plate reader (Wallac) at 450nm with a reference filter of 405nm.

All samples were assayed in duplicate on the same plate with a standard curve, blanks and low and high controls. All standards were within acceptable limits. The normal levels of serum cytokines accepted were <1.5pg/mL (IFN-γ), <4.6pg/mL (IL-4), (IL-10) and (IL-12), (Amersham Pharmacia cut-off values). The upper limit of normal for the CSF cytokines was calculated as the mean of the neurological disease controls plus 2 standard deviations. The cut-off levels of normal CSF cytokines was <0.6pg/mL (IFN-γ), <1.5pg/mL (IL-4), <0.5pgmL (IL-10) and <1.5pg/mL (IL-12) in this study.

2.19 Oligoclonal IgG bands

Isoelectric focusing can be used to separate proteins, such as immunoglobulins, according to the isoelectric points of the proteins. The isoelectric point is the specific pH at which a protein has zero net charge and is thus static at a particular point (focused). Protein separation occurs within a pH gradient which is generated by an electrophoretic field and is stabilised by using zwitterionic ampholytes. The ampholytes are soluble carrier molecules with a high buffering capacity. Resolution is controlled by the slope of the pH gradient which is produced by anodic and cathodic buffers.

The separated proteins were passively transferred on to a nitrocellulose membrane by placing a 1 kg weight on the top of the nitrocellulose membrane which had been placed on to the surface of the gel. Unoccupied protein binding sites are blocked with 2% milk proteins. Separated IgG clones were identified by incubating the blotted nitrocellulose

with diluted goat anti-human IgG antibody (Diasorin 80261). The presence of IgG clones which were polyclonal, monoclonal or oligoclonal were detected by the addition of a HRP conjugated anti-goat antibody (Dako P0406). Visualisation of the IgG pattern was carried out using an enzymatic detection method with the addition of the enzyme substrate, 3-amino-9-ethyl-carbazole (Sigma A5760) and hydrogen peroxide. Oligoclonal bands were assessed visually by comparing serum and CSF lanes (paired samples) and counting the number of bands in each lane (>2 bands=oligoclonal or homogeneous smear=polyclonal).

2.20 Cross-reactivity between anti-basal ganglia antibodies and streptococcus

To re-examine Husby *et al* hypothesis that ABGA reactivity could be removed by incubating with streptococcus I aimed to see if the antibody responses against GABHS isolated from a SC patient could be removed by incubating with brain tissue.

2.20.1 Streptococcal cultures

A UK patient with acute SC was throat swabbed and a M1 GABHS serotype cultured. A bacterial homogenate from this M1 serotype and a control streptococcal strain (M5) from a patient with PSGN were prepared for Western blotting by lysing the cell walls with bugbuster (CN biotechnology 70584) and preparing a homogenate. The streptococcal homogenates were incubated with LDS sample buffer and DTT using the same method as that for basal ganglia Western immunoblotting. The homogenates were run on a 2-D well 4-12% Nu-Page gel (Invtrogen NP0321) with a MW marker. The proteins were blotted onto nitrocellulose and probed using a 10 well plastic manifold.

2.20.2 Absorption experiments

The serum from the SC patient was investigated to ascertain if there was any specific antibody reactivity against the M1 serotype grown from the throat. The M5 serotype was used as a control. The serum was diluted 1/300 and incubating against both M1 and M5 overnight, after washing, 1/1000 diluted anti-human IgG conjugated with HRP was used to detect any antibody reactivity against the 2 streptococcal serotypes.

In a separate experiment, the 1/300 diluted SC patient's serum was pre-incubated with 100μg of: 1) basal ganglia homogenate and 2) human cerebellum homogenate in separate tubes at 37 °c for 1 hour. The final dilution of the serum was 1/300 in all instances. The tubes were centrifuged at 10,000rpm for 10 mins to precipitate immune complexes. The resulting supernatant was tested against Western immunoblots of the M1 and M5 serotypes. This was to examine if reactivity against the streptococcal proteins were absorbed out by incubating the serum with basal ganglia or cerebellum homogenates.

2.21 Identification of candidate antigens by two-dimensional protein electrophoresis

The basal ganglia homogenates underwent IEF and PAGE using 2 methods, and protein 'spots' were identified by staining the gel with either a silver stain. Electro-blotting on to nitrocellulose was used to allow for the probing of separated proteins in ABGA positive samples (SC, PANDAS and TS) and controls. Antibody binding to the 2-D gels was detected colorimetrically using 4-Chloro-1-Naphthol (Sigma chemicals C8890) or with a chemiluminescence kit (Perbio 34080). Any reactivity of ABGA against protein(s) on the nitrocellulose was used to identify the position of the protein in a stained gel (run at the

same time). Candidate protein 'spots' were cut out from the stained gels, enzymatically digested and the sequence identified by mass spectroscopy (MALDI-TOF) and searching a protein database such as Swiss-prot. The digestion of protein candidates and mass-spectroscopy was carried out by Dr Robin Wait at the Kennedy Institute of Rheumatology, Imperial College University, London.

2.21.1 Two-dimensional electrophoresis method: mini-gel system

Basal ganglia S1 fraction was diluted 1/2 with IEF sample buffer (Invitrogen LC5311) leaving a final concentration the same as that used in the basic Western immunoblotting methods, although this was adjusted if a higher or lower concentration was required to increase resolution of the gels and to avoid streaking.

First phase

- The anode and cathode buffer for the wet chamber was prepared and allowed to stand to remove air bubbles.
- 10-well IEF gel was placed into the inner portion of the tank and secured with wedges, the anode buffer was used to fill up the outside chamber.
- The prepared sample was pipetted into each well using 15μL, apart from well 1 which was filled with an IEF coloured marker (Invitrogen 39212-01).
- The gel was run for a total of 2 and 1/2 hours in a step gradient: 1 hour at 100V, 18mA and 2W; 1 hour at 200V, 18mA and 3.5W with 30 mins at 500V, 18mA and 9W.

- The gel was placed in fixing solution (TCA/SSA), (see appendix) for 30 mins to fix proteins and wash out pharmalytes which could give false signals on a stained gel.
- The gel was then briefly stained in Commassie and de-stained until clear (see appendix). The gel was then left for 1 hour in 20% ethanol to remove the acetic acid and shrink the gel slightly.
- A vertical strip was cut from the gel containing 1 column of proteins and placed in LDS and 25% ethanol for between 5 and 10 minuets.

Second phase

- A 2-D Nu-PAGE gel (Invitrogen NP0326) was prepared and the slice of gel was placed horizontally in the well and electrophoresed as previously described.
- The gel was either stained to reveal proteins or electro-blotted on to nitrocellulose to allow probing of the bound proteins by patient serum or monoclonal antibodies. The advantages of the mini-gel 2-D gels system is that small volumes of samples could be tested although at a cost of reduced separation capacity due to the size of the gel and the available pH ranges.

2.21.2 Two-dimension electrophoresis: immobiline gel system

The limitation of the mini-gel system was that only one pH range could be used in the first dimension (pH 3-10) and the size of the gels meant that separation between protein 'spots' was not always complete. An immobiline gel strip system such as a pH 3-10 range (Amersham Pharmacia 17-1234-01) was used to increase protein separation due to the larger size of the strips (18x18cm IEF gel strips) and overlapping pH gradients were

available to increase focusing (i.e.; 3-10, 9-11 and 3-5). The basal ganglia homogenate was first diluted in solubilisation buffer (see appendix). The immobiline buffer (pH range dependant on gel used) was then added (Amersham Pharmacia 17-6000-87 for pH 3-10) and the sample thoroughly mixed.

First phase

- The basal ganglia proteins, diluted in solubilisation buffer were used to reconstitute the immobiline strips of the appropriate pH range (stored at -20° C).
- The IEF strips were placed in a dry strip holder (Amersham Pharmacia 80-6371-84) and 350µL of the basal ganglia solution was pipetted on to each strip which was covered with mineral oil (Amersham Pharmacia 17-1335-01) to prevent evaporation and contamination.
- The strips were left overnight to reconstitute.
- Each strip was aligned in the multiphor II electrophoresis system (Amersham Pharmacia 18-1018-06).
- The strips were electrophoresed under mineral oil to prevent drying (Amersham Pharmacia 17-1335-01) using the following conditions: 1 min at 500V, 2mA, 5W; 1 hour 30 mins at 3500V, 2mA, 5W and 8 hours at 3500V, 2mA and 5W.
- After the run the strips were laid on blotting paper to remove excess moisture and prepared for the second SDS-PAGE separation.
- The strips were equilibrated in two buffers (see appendix) and left for 5 mins with gentle rocking. After a gentle rinse in double distilled water the strips were placed in the same stock solution for 5 mins. This contained iodoacetamide (Sigma I-

1149) which alkylates thiol groups, lessening streaking of proteins during the second phase of electrophoresis.

Second Phase

- An equilibrated IEF strip was placed horizontally, face-down onto an 18x18 cm SDS gel (Pharmacia 80-6002-21) with an additional strip on the left hand side of the gel soaked in molecular weight marker (Invitrogen LC5925).
- The anodic buffer strip (Amersham Pharmacia 17-1342-01) was positioned 1 cm in from the edge of the SDS gel near the positive electrode. The cathode buffer strip (Amersham Pharmacia 17-1342-01) was placed 1 cm from the edge of the gel near the negative electrode.
- To prevent water from 'streaking' the proteins, squares of blotting paper were placed underneath both ends of the IEF strip to allow water to move away from the region of protein separation.
- The electrodes were aligned with the buffer strips and the lid placed on the electrophoresis tank (Amersham Pharmacia 18-1018-06).
- The gel was run in 2 steps: 120V, 20mA, 30W for 40 mins, after which the IEF strip was removed. Step 2 was 600V, 50mA, 30W and 1 hour or until the bromophenol blue (Amersham Pharmacia 17-1329-01) front reached the anodic buffer strip.
- The gel was either stained in Commassie or electro-blotted on to nitrocellulose membrane (Sartorius WP4HY320F2).

2.22 Immunoprecipitation

In experiments to purify out the most common basal ganglia antigens (40, 45, 60 and 98 kDa) recognised using Western immunoblotting, a Mammalian cell immunoprecipitation kit was purchased (Perbio 45225). The system is designed to utilise small concentrations of antibody and antigen source and is carried out in microcentrifuge tubes (2ml) containing a spin cup (semi-permeable membrane), (Perbio 45225).

Basic method

- Immobilised protein G, gel slurry (50% with buffer) was pipetted (0.4mL) into a microcentrifuge tube containing the spin cup and was centrifuged at 10,000 rpm for 1 min to remove excess buffer and leave protein G gel in the cup.
- The protein G gel was washed with binding buffer for 2 mins by inverting the tube and centrifuged at 10,000 rpm for 1 min to remove excess buffer.
- Purified IgG (500 μg) from a patient with acute SC (ABGA positive) was pipetted into the tube and left to incubate with gentle rocking at 4°C overnight to allow antibody to bind to the immobilised protein G gel.
- The tube was allowed to reach room temperature and was centrifuged at 10,000 rpm for 1 min to remove unbound antibody. The flow-through was collected and its protein concentration determined to calculate the relative amount of antibody binding.
- An antibody cross-linker (disuccinimidyl suberate) was dissolved in dimethyl sulfoxide (DMSO), (Sigma D2650) and pipetted immediately into the spin cup. The antibody linker avoids immunoglobulin contamination (from the bound antibody) in the final elutes and preserve the bound antibody for future

immunoprecipitation. The linker was incubated with the protein G, gel for 1 hour at room temperature with gentle mixing.

- The tube was then centrifuged at 10,000 rpm for 1 min to remove excess fluid and washed 4 times with quenching buffer to remove DMSO.
- Elution buffer (500 µL) was then added and the tube gently mixed by inverting 10 times. The tube was centrifuged and the liquid flow-through collected. The elution step was repeated 4 times and the liquid collected into numbered tubes. This step removed the excess antibody linker and any contaminants, leaving behind the protein G-bound antibody.
- Human basal ganglia homogenate was mixed with an equal volume of binding buffer and 500 µL of this solution was added to the washed spin cup containing the protein G, gel. The homogenate was incubated in the cup overnight at 4°C with gentle rocking.
- The tube was then allowed to come to room temperature and centrifuged at 10,000 rpm for 1 min and the liquid (unbound homogenate) removed.
- Washing buffer was added and the gel washed by inverting the tube 10 times, centrifuging at 10,000 rpm for 1 min and collecting the flow through. This was repeated twice to wash off unbound antigen.
- The elution buffer (Perbio 45225) was then added (190 µL) to the tube and mixed with the protein G, gel by inverting the tube 10 times.
- The tube was centrifuged at 10,000 rpm for 1 min and the liquid flow-through collected. This elution step was repeated a further 4 times and the flow through

collected. The spin cup was washed with buffer several times and left in 500µL of washing buffer with 0.1% sodium azide and stored at 4°C for future use.

- The protein concentrations of the flow-through from antibody, antibody cross-linker, antigen binding and final protein elutions were calculated.

PAGE gel of eluted protein

The final elutes were prepared for PAGE analysis by adding LDS buffer containing 0.05M DTT and heated to 65°C. The elutes were electrophoresed in a 10 well 4-12% bis-tris gel (Invitrogen NP0321) gel to assess the antigen(s) collected from the immunoprecipitation method. The gels were stained with silver and the pattern and number of bands (if any) recorded for each elute. The subsequent Western blots were probed with ABGA positive antibodies (40, 45, 60, 98 kDa) to assess whether the reactive antigen was immunopreciptated from the crude basal ganglia homogenate and was present in a particular elute. Specific binding to the eluted antigens was compared to that from Western blotting and antibody probing of crude basal ganglia homogenate. Any reactive antigens were cut out of the gel and identified using mass spectroscopy. Separate immunoprecipitation was carried out for each of the known basal ganglia antigens (positive sera with reactivity against one of the antigens alone 40, 45, 60 and 98 kDa).

As the recovered protein from immunoprecipitation or 2-D electrophoresis may have been too low for peptide identification. A commercial centrifugal filter device (YM-100, Milipore 42412) was used with a membrane specific for a particular molecular weight cut-off. This stopped the loss of proteins with molecular weights >10,000 kDa. Recovery of proteins after concentration was reported to be 95% (Millipore, 42412) with a

concentration factor as high as 100% depending on how long centrifugation was required and the volume of buffer used to re-suspend the protein.

Briefly, a Microcon sample reservoir was inserted into a 1.5mL microcentrifuge tube. A maximum of 0.5mL of sample was pipetted into the sample reservoir and centrifuged at 14,000g for 10 mins in a microcentrifuge (Whatman Force-7). The sample reservoir was then removed and placed upside down into a fresh microcentrifuge tube. The tube was then centrifuged at 10,000g to transfer the concentrate into the bottom of the new microcentrifuge tube. The sample reservoir was then removed and the concentrated sample was ready for use or storage. The recovered proteins were compatible with PAGE and mass-spectroscopy

2.23 Anti-human neuron-specific enolase in controls and acute Sydenham's chorea

To assess neuron-specific enolase (NSE) as a candidate autoantigen in post-streptococcal movement disorder syndromes, neurone-specific enolase antigen was purchased commercially (Sigma N-4773). The antigen was prepared for Western immunoblotting using the same procedure used for basal ganglia homogenates. Due to the cost of this antigen a total of 5μg of protein was loaded per well (approx 0.5μg per lane).

Six controls with movement disorders and streptococcal disease were used as controls the diagnosis were: DYT1 positive dystonia; Huntington's chorea; "idiopathic" Parkinson's disease; PSGN; Streptococcal pharyngitis and Multiple sclerosis. Samples from 6 patients

with acute SC who had not been tested previously for ABGA were kindly provided by Dr. F. Cardoso, Federal University of Minas Gerais, Brazil. All samples were diluted 1/300 and tested using the same method used for the basal ganglia homogenate Western immunoblotting method. For positive control, a commercial antibody (goat polyclonal) specific for human NSE (C-terminus specific), (Santa Cruz, sc-7455) was tested at a 1/1000 dilution on Western immunoblotting and detected using a rabbit anti-goat HRP-labelled secondary antibody (Dako, P-0449).

2.24 Functional effect of ABGA on enolase enzymatic activity

This investigation was designed to see whether the antibodies could alter the normal glycolytic enzyme activity of enolase. As enolase is one of the glycolytic enzymes (conversion of pyruvate to lactate) the production of NAD+ from NADH+ was used to assess the glycolytic NSE activity in the presence of purified IgG from subjects with ABGA and IgG from neurological controls (Parkinson's disease).

2.24.1 Enolase and IgG purification

Enolase was purified from human brain using ammonium sulphate and Fluid Phase Chromatography (FPLC) and was kindly provided by Dr Russell Dale, Institute of Child Health, Great Ormond Street, London. Serum samples were run through a protein A column using FPLC to purify IgG (Amersham Pharmacia 17-042-03), (see appendix).

2.24.2 Alterations in enolase activity with anti-enolase and control IgG

The enolase activity was determined by measuring the transformation of $NADH^+$ to NAD^+, a method previously described (Pancholi *et al.*, 1998). The enzyme reaction was performed using purified NSE from rat brain, kindly provided by Dr Russell Dale, Institute of Child Health and Neurology, Great Ormond Street Hospital. The enzyme activity assay took place in a microtitre plate, by first adding NSE to the reaction substrate (see appendix). The activity of the enzyme was measured by adding 100 µL of NSE and recording the change in absorbance at 340nm (conversion of $NADH^+$ to NAD^+) using a plate reader (Wallac multi-label counter). This was carried out by taking an absorbance reading in 30 second intervals until no change in absorbance could be detected. The decrease in extinction at 340nm was due to the increase in production of NAD^+ from $NADH^+$ and the results plotted onto an Excel (Microsoft) spreadsheet for further analysis.

Anti-NSE positive serum was put through a protein A column to purify IgG. The effect of anti-NSE positive IgG was analysed by incubating it with NSE using the same antigen and antibody dilutions as used for analysis of ABGA (1/300 serum dilution and 30 µg of protein). The IgG was incubated with NSE using gentle rocking at room temperature. Every hour, 100µL of the incubated NSE was pipetted from the stock solution and the NSE activity measured. This was carried out until a maximum of 12 hours incubation had occurred. Alterations in the enzyme activity were assessed by recording the effect of the antibody incubations on the decrease in absorbance at 340nm compared to baseline enzyme measurement (without IgG). To control the study and to study any deterioration

in the NSE activity over time, 5 IgG control samples from patients with idiopathic Parkinson's disease were tested. A control sample was tested in parallel using the same antigen source and at the same time as the anti-NSE antibody experiment.

2.25 Statistical analysis

All statistical analyses were done using SAS software (SAS Institute, Inc., Cary, North Carolina, USA). Streptococcal serology (ASOT and DNAse B) and ABGA, cytokine ELISA absorbance data were compared using the non-parametric 2 sample exact Wilcoxon rank-sum test (Mann-Whitney). Comparison of the numbers of samples with raised streptococcal serology was carried out using the Fisher's exact test. Positive ABGA Western immunoblotting was compared in each subgroup using Chi-square tests. All confidence intervals quoted refer to 95%.

2.26 Conclusions

ELISA, Western immunoblotting and IF was used to detect ABGA in serum samples from patients with SC, PANDAS and TS. Streptococcal infection was determined by surrogate serology and clinical presentation. A proteomic method was chosen to identify putative autoantigens in these syndromes.

3 Results of Indirect immunofluorescence

3.1 Indirect Immunofluorescence

Introduction

The Indirect Immunofluorescence work in this thesis was carried out with the kind assistance of Neuropathology, although, routine requirements of the laboratory meant access to microscopes and other equipment was limited. Immunohistochemistry could also have been performed as this is a standard method for detecting anti-neuronal antibodies.

Unfortunately, no grant monies were secured for conducting a large histopathological study of anti-basal ganglia antibodies. Therefore only these pilot studies using indirect immunofluorescence were performed. However, the aim of this thesis was to attempt to identify and characterise the antigens using proteomics. A separate series of studies is required to investigate antibody binding by histochemistry.

3.2 Sagittal rat brain sections

Introduction

Frozen sections of rat brain sagittal sections were purchased from Novagen, Germany. Four patients with Sydenham's chorea and 4 normal controls were diluted 1/50 and 1/500 and tested by IF in accordance with a commercial suggestion for detected anti-neuronal antibodies in paraneoplastic disease using indirect immunofluorescence (The Binding Site, UK), for routine diagnostic purposes (Figure 3-1).

A commercial monoclonal antibody which recognised grey matter (basal ganglia areas), dopamine D2 receptor (Autogen Bioclear), (Secondary antibody FITC labelled rabbit anti-goat immunoglobulins, Dako) was tested. Cerebellum was identified by testing just 1 paraneoplastic Hu sera from a patient with SCLC and neurological disease.

IgG binding to the section was identified using FITC labelled anti-Human IgG (Dako). This was to assess grey matter area staining of SC samples in comparison to the D2 receptor identified area and cerebellum in comparison to one Hu positive serum.

More samples were not tested due to the expense of these sections and the negative results of SC samples tested (Figure 3-1).

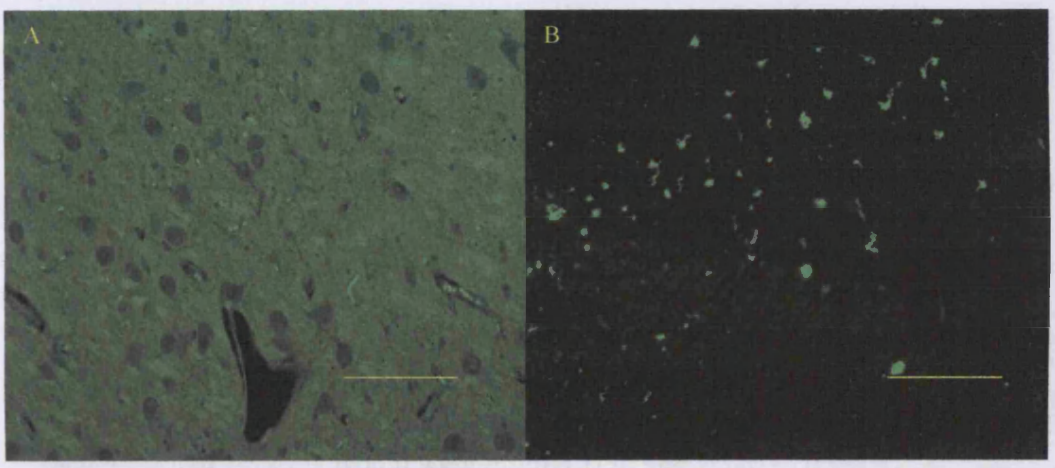
Results from sagittal sections

Normal controls (Figure 3-1a) did not bind specifically to any brain structure. Secondary antibody against Human IgG (fab) FITC labelled (Dako) did not bind to any brain structure. Photograph of this had low contrast and brightness and did not print adequately which is why they are not shown.

In comparison the D2 receptor antibody (Figure 3-1b) identified deep grey matter neurones. The commercial anti-enolase monoclonal antibody bound to neurones within this area, but, there was neuronal staining across the section with this enolase antibody (Figure 3-2b) and was not confined to the D2 stained area. There was no staining of the D2 area with the 4 SC samples tested either at 1/50 or 1/500.

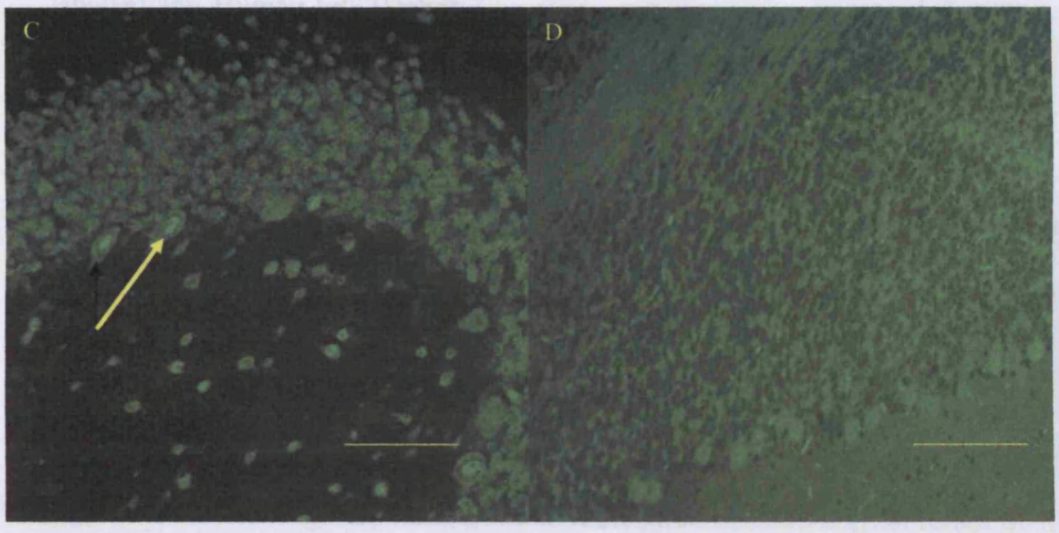
A patient with PNS and Hu positive serum bound to Purkinje cells within the cerebellum as expected (Figure 3-1c). There was no other neuronal or glial staining evident. The 4 patients with SC did not appear to bind at either 1/50 or 1/500 to Purkinje cells (Figure 3-1d).

Figure 3-1 Sagittal Rat Brain slices of 4 normal controls, 1 commercial D2 receptor, 1 Hu antibody positive and 4 Sydenham's chorea samples against cerebellum and grey matter areas



A: Normal (non-diseased) control diluted 1/50, IgG did not stain any structure mag x200

B: Commercial dopamine D2 receptor antibodies 1/500 identified grey matter neurones in sagittal Section, mag x200



C: Hu sample diluted 1/500 had Positive staining of Cerebellum Purkinje cells (arrow) mag x200

Key: Yellow bar 5µm

D: Sydenham's chorea sample diluted 1/50 did not bind to Purkinje cells mag x200

3.3 Results of sagittal section stained with four Sydenham's chorea samples and four normal controls to assess neuronal staining within the cerebral cortex.

Introduction

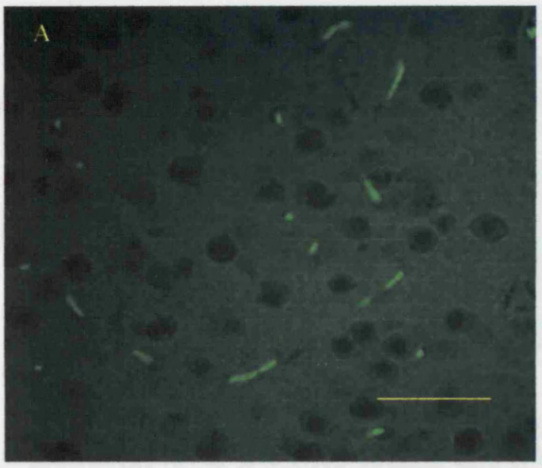
Husby's original report of basal ganglia antibodies suggested that the antibodies also bound to the cerebral cortex (Husby *et al.*, 1976).

The same batch of slides were also incubated with 4 acute Sydenham's chorea samples and 4 normal controls which were diluted 1/50 and 1/500 in 0.9% saline and incubated with the section for 30 minutes. IgG binding to the section was identified using FITC labelled anti-Human IgG (Dako).

Results

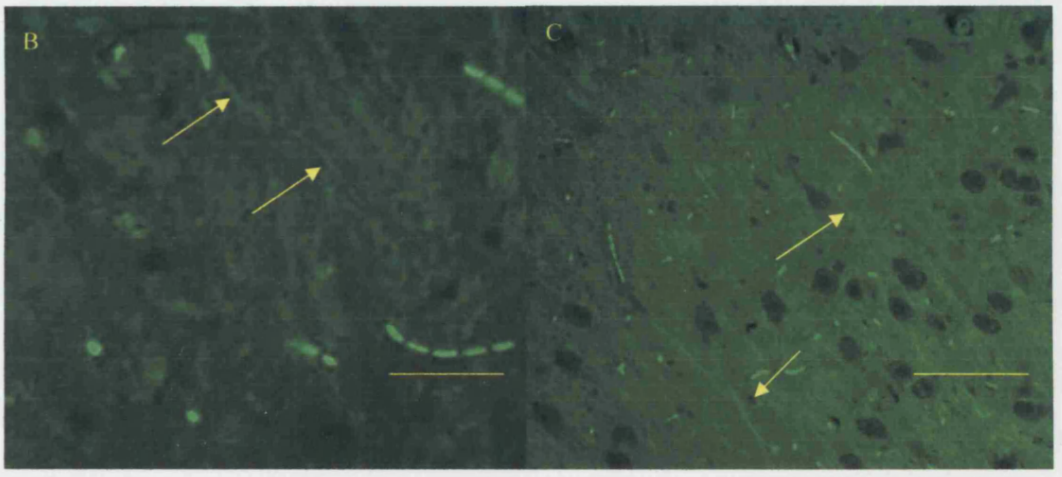
Sydenham's samples diluted 1/500 were all negative (4 samples). There was no binding to neurones or anything else. Patients with Sydenham's chorea only bound weakly to neurones at a dilution of 1/50 (Figure 3-2c). The 1/50 binding was similar to that seen with the commercial enolase antibody which was axonal, cytoplasmic staining (Figure 3-2b). Neuronal staining was weak and distributed throughout the brain rather than in one distinct area.

Figure 3-2 Commercial anti-neuron specific enolase antibody diluted 1/500 and tested against rat cerebral cortex and ten patients with Sydenham's chorea and 10 normal controls diluted 1/50



A: Normal control diluted 1/50 against rat brain section.

There is no obvious staining, mag x200



B Commercial NSE P-2 antibody diluted 1/500 against rat brain sagittal section
Identified neurones, mag x400
Key: Yellow bar 5μm

C: Sydenham's chorea serum diluted 1/50 against sagittal section binds only weakly to isolated neurones in the cortex, mag x100

Conclusions

Sydenham's chorea samples only had weak IgG binding to neurones, unlike, paraneoplastic antibodies there was no Purkinje cell staining and the concentration of antibody was significantly lower than that expected in paraneoplastic syndromes (1/500-1/5000). This means that anti-neuronal antibodies in SC are weak and possibly of no significance. There was no obvious staining like that seen with the D2 receptor, although it was assumed that this antibody recognised the basal ganglia area in rats which may not be the case. Neuronal-like staining was only diffuse rather than to one distinct area. Therfore basal ganglia antibodies do not appear to be specific to the basal ganglia in rats.

However, these negative results might be influenced by species differences in antigen concentration, localisation and presentation. The original publications of basal ganglia antibodies used human tissue (Husby *et al.*, 1976). Therefore primate and human brain were studied.

3.4 Indirect immunofluorescence against monkey cerebellum, cerebrum and mouse stomach

Introduction

As most accepted anti-neuronal antibodies bind to cerebellar neurones the post-streptococcal neurological disease patients were tested for the typical paraneoplastic antibodies. A typical, routine method was used with primate (monkey) tissue containing cerebellum, cerebrum and mouse stomach (The Binding site, UK). This is a method commonly used for detecting anti-neuronal antibodies by IF in paraneoplastic syndromes (PNS), (The Binding Site, UK). One patient positive for Hu and 1 with Yo were used for controls, (Figure 3-3c and e).

Ten acute SC and 5 normal controls were diluted both 1/50 and 1/500 (dilutions suggested by manufacturer, The Binding Site, UK) and tested against cerebellum, Cerebrum and mouse stomach sections (The Binding Site, UK).

Results

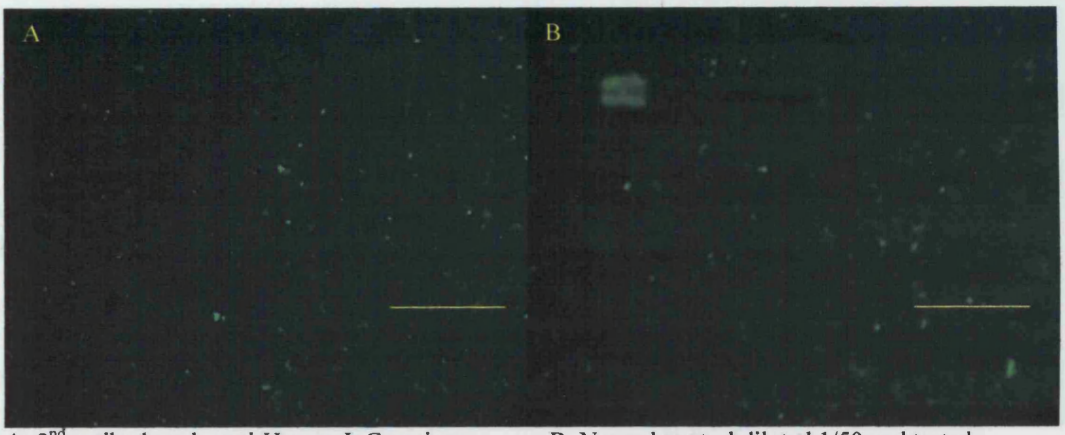
None of the SC patients had IgG binding to the cytoplasm of monkey Purkinje cells, although one patient had nuclear binding (Figure 3-3f). All patients were negative for paraneoplastic antibodies against cerebellum. The known positive Hu and Yo samples gave expected staining of cerebellar Purkinje cells (Figure 3-3c and e). None of the normal controls had any neuronal staining the background fluorescence was not different from that of sections incubated with just secondary antibody alone (Figure 3-3a and b).

There was staining of neurones in the cerebellar granule layer, this neuronal staining was identified by testing the same sections with a commercial anti-NSE antibody. The pattern of the staining was cytoplasmic and appeared to be similar with anti-NSE antibody (Figure 3-4a) and SC samples (Figure 3-4 b and c). This staining was weak and confined to scant neurones only.

There was scattered staining of neurones with axonal like pattern in the cerebral cortex of monkey section with all ten SC samples at a dilution of 1/50. Only 2/10 (20%) had weak staining of neurones at a serum dilution of 1/500. The pattern of IgG staining was similar to that seen with commercial NSE antibody suggesting the antibodies bind to axonal cytoplasm. There was no evidence of membranous staining, although, this could be masked by cytoplasmic staining.

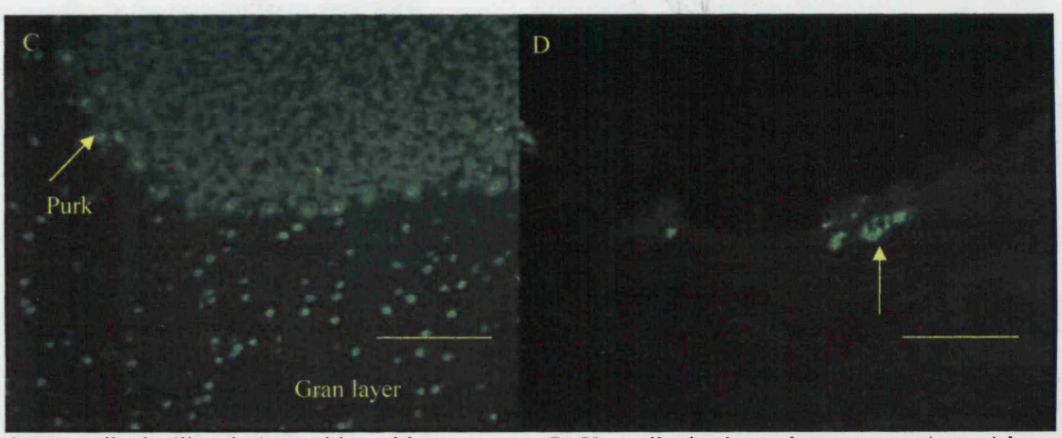
Double staining a section with a SC serum diluted 1/50 with anti-human IgG FITC and a commercial glial fibrillary acid protein (GFAP) monoclonal antibody, (Sternberg Monoclonals) with anti-mouse TRITC identified glial cells (Figure 3-4c and d). The SC samples clearly had different staining to the GFAP antibody. The anti-neuronal staining in SC was similar to that seen with the commercial anti-NSE antibody showing that SC IgG binds to neurones albeit at low titre.

Figure 3-3 Monkey Cerebellar and mouse stomach sections



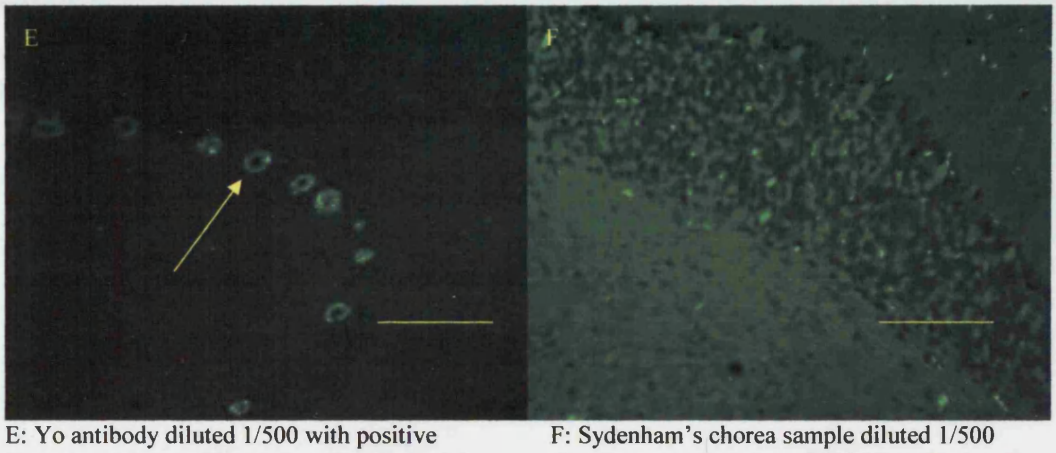
A: 2nd antibody only anti-Human IgG against monkey cerebellum. Background staining only, mag x200

B: Normal control diluted 1/50 and tested against monkey cerebellum. Background staining only, mag x200



C: Hu antibody diluted 1/500 with positive Purkinje cells and granular neurones Nuclear staining, mag x200

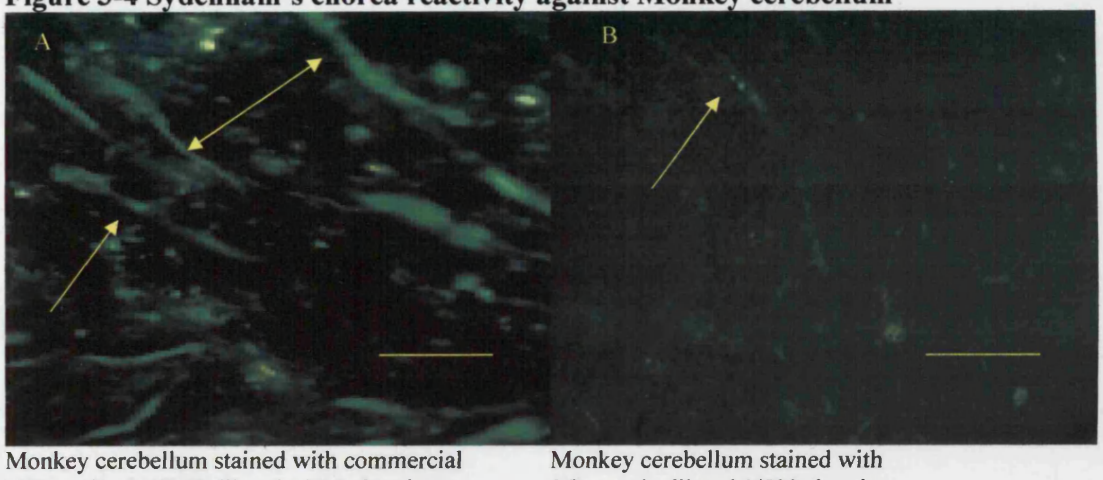
D: Hu antibody also stains neurones (arrow) in mouse stomach myenteric plexus, mag x200



E: Yo antibody diluted 1/500 with positive Granular staining of Purkinje cells, (arrow) Mag x200, **Key: Yellow bar 5µm**

F: Sydenham's chorea sample diluted 1/500 showing some staining of Purkinje cells nuceli mag x200

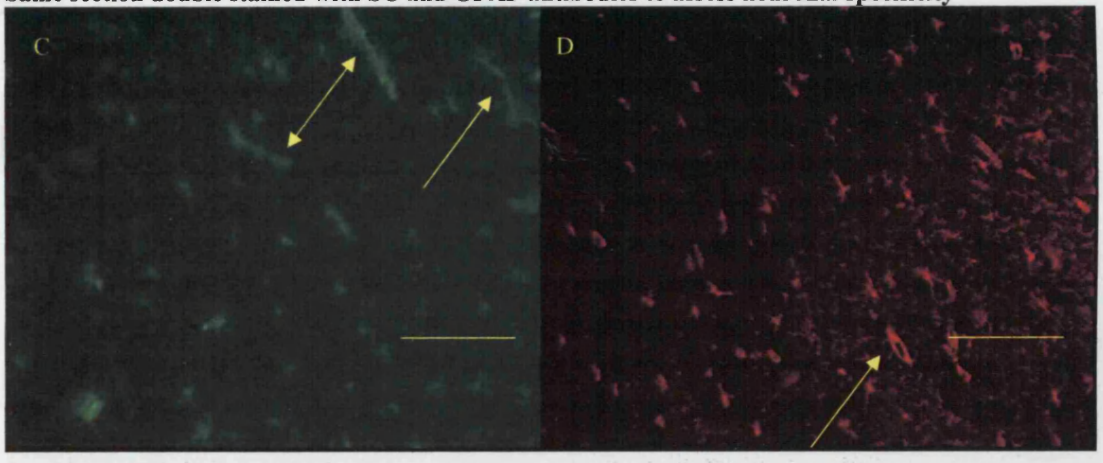
Figure 3-4 Sydenham's chorea reactivity against Monkey cerebellum



Monkey cerebellum stained with commercial NSE antibody (P-2) diluted 1/500 showing predominant cytoplasmic localisation (arrows) of staining in the granular layer, mag x400

Monkey cerebellum stained with SC sample diluted 1/500 showing similar pattern to NSE with weak cytoplasmic localisation (arrow) of staining in the granular layer, mag x400

Same section double stained with SC and GFAP antibodies to assess neuronal specificity



Monkey Cerebrum with Sydenham's chorea Sample diluted 1/50 showing weak Axonal Staining, mag x400

Monkey Cerebrum with anti-GFAP antibody diluted 1/1000 showing glial cell staining in the same section, mag x400 **Key: Yellow bar 5μm**

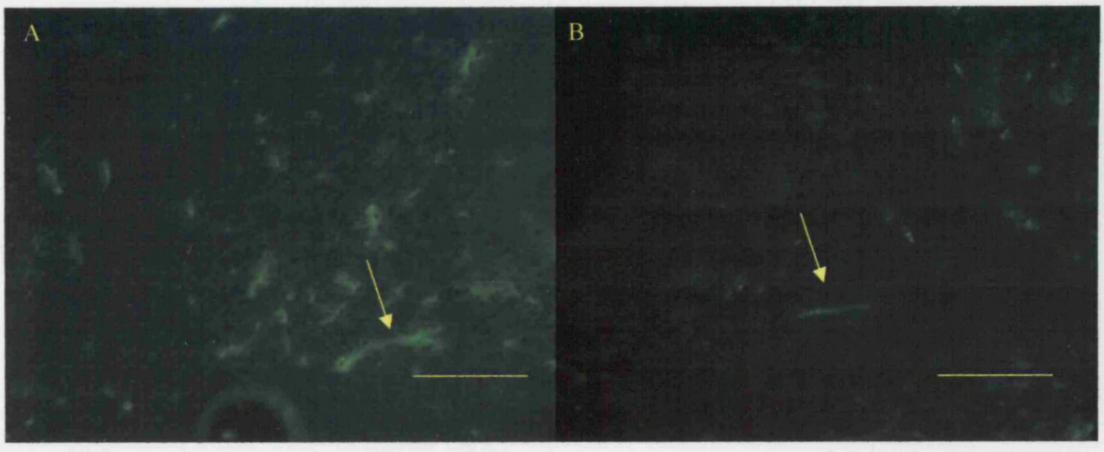
3.5 Tourette's syndrome and patients with PANDAS have a similar anti-neuronal like staining pattern using monkey tissue but only at 1/50 dilution

Five patients with Tourette's and PANDAS were tested against the same sections as SC studies (Figure 3-5). The five patients had been identified as possible positive samples using Western immunoblotting.

Results

There was a similar pattern of neuronal staining with axonal, cytoplasmic predominance. The optimum staining was again only at a dilution of 1/50.

Figure 3-5 Tourette's syndrome and PANDAS patients tested against monkey sections



Monkey Cerebellum with Tourette'syndrome Sample diluted 1/50 Showing neuronal (axonal) mag x400
Key: Yellow bar 5µm

Monkey Cerebellum with PANDAS sample diluted 1/50 Showing neuronal staining mag x400

Conclusion

Antibodies in SC were proposed to be basal ganglia specific (Husby *et al*, 1976) although the authors suggested weaker staining in the cortex. Therefore these results are consistent with Husby's reported findings (Husby *et al.*, 1976). However, neuronal staining was also present in the cerebellar granular layer, these cells are easily cultured so live cells assays could be performed using IgG from SC patients to investigate whether these antibodies have any functional effects. Repeating Husby's original study using human basal ganglia sections was also done.

There appears to be low titre anti-neuronal like staining in post-streptococcal movement disorders. However, the staining is cytoplasmic and therefore unlikely to constitute a functional autoantibody disorder as no membranous distribution is evident. The antigen(s) are likely to be soluble and might be of little consequence. However, in the absence of control positivity, these antibodies might be a marker of disease. For example most anti-neuronal antibodies are non-functional but, are very useful for the diagnosis of autoimmune disorders mediated by other processes such as aberrant T-cells.

3.6 Husby's method to detect anti-basal ganglia antibodies against human tissue by indirect immunofluorescence in 4 Sydenham's chorea samples

Introduction

The suggested method for detecting ABGA (Husby *et al.*, 1976) was by counting and grading positive stained neurones in human basal ganglia using ½ diluted sera Husby *et al,* 1976. Sections from 3 different normal striatums were tested in SC, PANDAS and TS, normal controls and secondary antibody only was set up to investigate if artefact staining of neurones was present.

I repeated the published method of Husby, (Tables 3-1 and 3-2), using 4 samples of patients with acute SC. All the experiments were carried out using the same block of non-diseased human basal ganglia. These sections were kindly cut by Linda Kilford, Queen's square Brain bank for Neurological diseases.

Results

I repeated the method of Husby exactly, but, grading the intensity of fluorescence as a method of assessing positivity was very unreliable. It was very difficult to see the difference between positive neuronal staining and background.

Conclusion

This method is too unreliable to interpret results. Kiessling *et al* used grading of fluorescence to investigate ABGA so I repeated this next, for methodological and comparison purposes.

3.7 Grading of indirect Immunofluorescence results in 4 Sydenham's chorea samples diluted 1/2 using Husby's method

Four acute SC samples were tested in triplicate and the following scheme was used to grade the neuronal staining: Neg (negative), + (weak positive), ++ (positive), +++ (strong positive). This method of assessment produced inconsistent results (Table 3-1). This method proved difficult due to high background fluorescence.

Table 3-1 Grading of indirect immunofluorescence of samples diluted 1/2

Sample	Test	Test	Test
Sample 1	+	++	+
Sample 2	+++	+	+
Sample 3	++	+	++
Sample 4	+	+	Neg

Positive neurones were counted in 1 microscopy field at low power (original magnification x200) in the middle of the caudate head using the same ABGA positive sample. This resulted in differences (mean difference 30.3 neurones (range 28-33) in the

numbers of positive neurones counted in 1 field (Table 3-2). Testing tissue sections from different blocks of striatum with the same sera also produced different positive neuron counts (mean difference 30.5 neurones (range 17-49)), despite testing the same SC serum (Table 3-3). This appeared to be due to variations in section quality, since the samples were tested in an identical fashion.

Table 3-2 Duplicate positive neurones counted in one microscope field

Sample	Counted positive neurones	Counted positive neurones	Counted positive neurones	Counted positive neurones	Mean neurone count (range)
Sample 1	40	33	59	28	40 (28-59)
Sample 2	15	39	29	48	32.8 (15-48)
Sample 3	40	51	45	69	51.3 (40-69)
Sample 4	51	42	68	40	50.3 (40-68)

Table 3-3 Testing the same sample against sections from different blocks

Sample	Counted No of positive neurones (Block 1)	Counted No of positive neurones (Block 2)	Percentage difference in counts
Sample 1	39	70	56%
Sample 2	25	50	50%
Sample 3	40	89	45%
Sample 4	51	68	75%

Conclusion

Grading and counting neurones was unreliable and neat or ½ dilutions of serum gave high background. Therefore a higher dilutions of sera: 1/25, 1/50 and 1/500 were investigated, to allow any discrimination between positive or negative sample to be better defined.

Serial dilutions other than 1/50 and 1/500 preparations of each sample was not done routinely due to limitations in numbers of tissue sections and the variations in section quality (Tables 3-2 and 3-3). Poor tissue preservation might be a cause for the variability in results, itself influenced by time of storage of brain tissue post death.

Serial titrations would of course show the average expected titre of ABGA. However, from earlier experiments, staining is not present at dilutions of only 1/500, so ABGA were assumed to be of low concentration. However, titrating samples to endpoint could be done as some patients might have high titre antibodies which influence clinical presentation or outcome.

3.8 Altered method using a 1/50 dilution of serum improved immunofluorescence results in 10 normal and 10 rheumatic fever controls

Introduction

I found that diluting the serum 1/50 produced clearer results compared to Husby's methods as background fluorescence was reduced (Figure 3-6).

Results

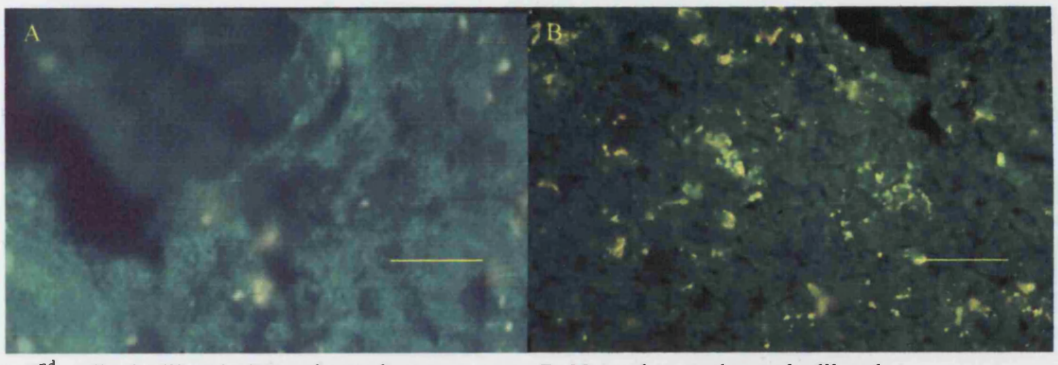
Background fluorescence such as lipofuschin granules was present in secondary antibody tested against the basal ganglia section without serum, but there was no obvious anti-neuronal staining (Figure 3-6a).

It was found that 10 normal control samples tested did not produce any specific staining of neurones or accessory cells at a dilution of 1/50 (Figure 3-6b-d).One of the RHF controls (10%) was positive for weak axonal-like staining (Figure 3-e).

Conclusion

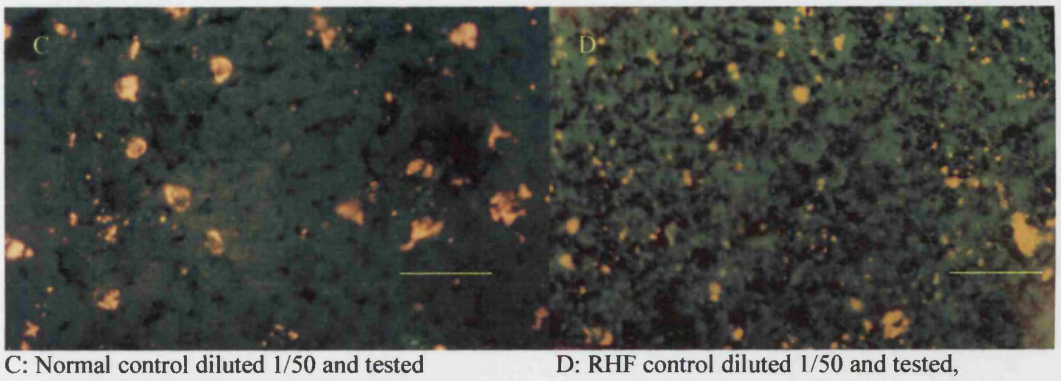
Therefore neuronal-like staining might be present in some cases of RHF without obvious chorea. Normal controls did not appear to have neuronal-like staining.

Figure 3-6 Typical results from control sample from a control with post-streptococcal glomerulonephritis tested 1/50 against human caudate and putamen section



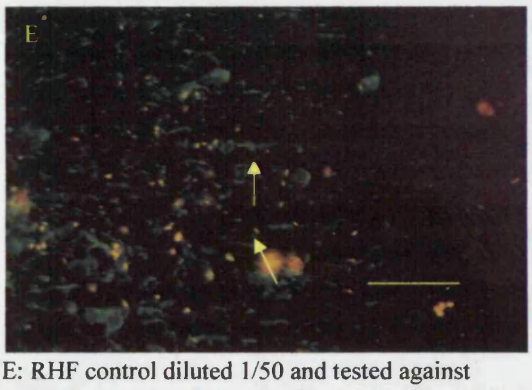
A: 2nd antibody diluted 1/30 and tested against Human basal ganglia section No specific staining. Lipofuschin granules, mag x200

B: Normal control sample diluted 1/50 and tested against human basal ganglia section. High background but no specific staining. Lipofuschin granules, mag x200



C: Normal control diluted 1/50 and tested against human basal ganglia. No specific staining, Lipofuschin granules, mag x200

D: RHF control diluted 1/50 and tested, against human basal ganglia. No specific staining Lipofuschin granules, mag x200



E: RHF control diluted 1/50 and tested against against human basal ganglia. Weak specific staining, (arrows), mag x200

Key: Yellow bar 5µm

3.9 Anti-basal ganglia antibody results in a large Sydenham's chorea cohort using indirect immunofluorescence and 1/50, single point dilution of sera

Introduction

Using the developed IF method (1/50 dilution of serum) described earlier 34 SC, 24 persistent SC, 20 normal controls and 20 RHF samples were diluted 1/50 and tested against human basal ganglia.

Results

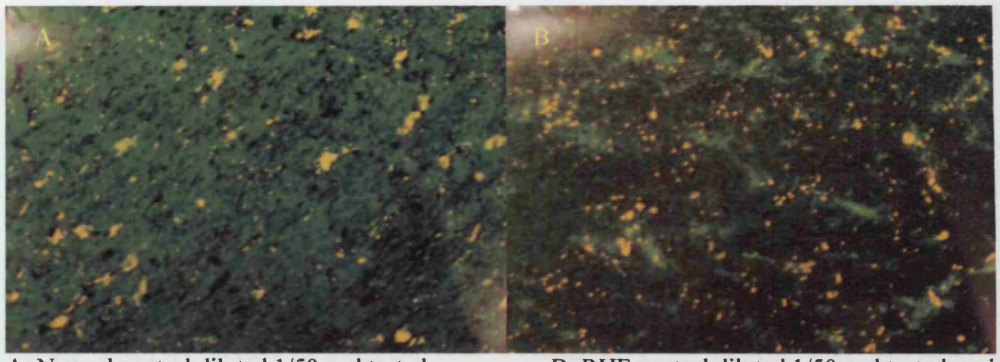
No normal control showed any specific binding (Figure 3-7a). Eighteen of the 20 RHF cases had no specific binding (Figure 3-7b), but 2 of the RHF controls had axonal-like staining (Figure 3-6e).

In acute SC 97% (33/34) had IgG binding to axons (Figure 3-7 b-e and Figure 3-8). Binding dropped to 63% (15/24) in persistent SC, but the staining pattern was similar to acute SC (Figure 3-7f). The acute SC and persistent SC groups both had increased IF positivity compared to both RHF ($p=<0.01$) and normal controls ($p=<0.01$). There was a higher percentage of positivity in acute compared to persistent SC ($p=<0.01$).

Conclusion

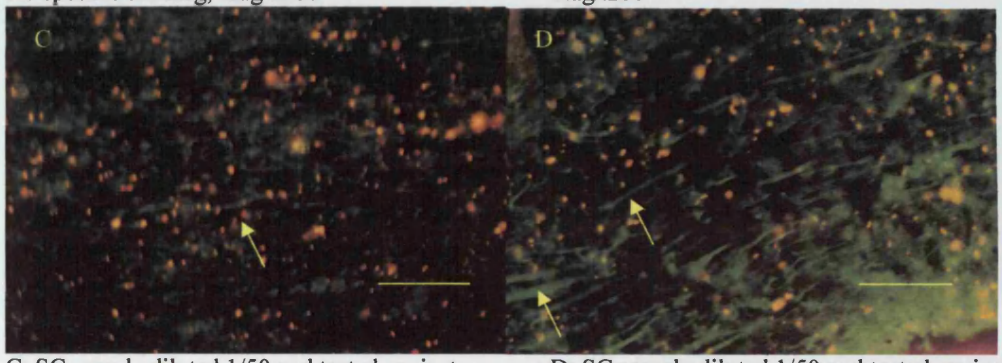
The binding appeared to be predominantly cytoplasmic though it was difficult to establish whether membranes were also stained. There were some yellow precipitates of lipofuschin which is not uncommon when using IF methods on human tissue (Figure 3-6 and 3-7).

Figure 3-7 Indirect Immunofluoresence of Sydenham's chorea, rheumatic fever and normal controls diluted 1/50 and tested against human basal ganglia



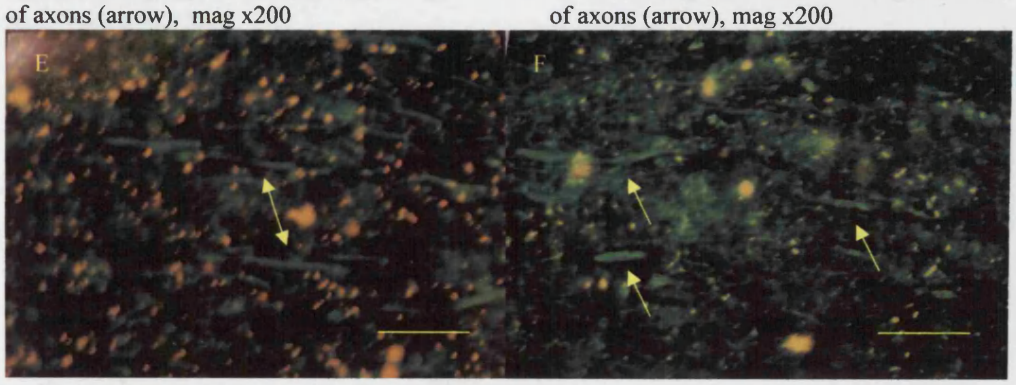
A: Normal control diluted 1/50 and tested Against human basal ganglia tissue. No specific staining, mag x200

B: RHF control diluted 1/50 and tested against Human basal ganglia. No specific staining, magx200



C: SC sample diluted 1/50 and tested against Human basal ganglia tissue. Weak staining of axons (arrow), mag x200

D: SC sample diluted 1/50 and tested against human basal ganglia tissue. Strong staining of axons (arrow), mag x200

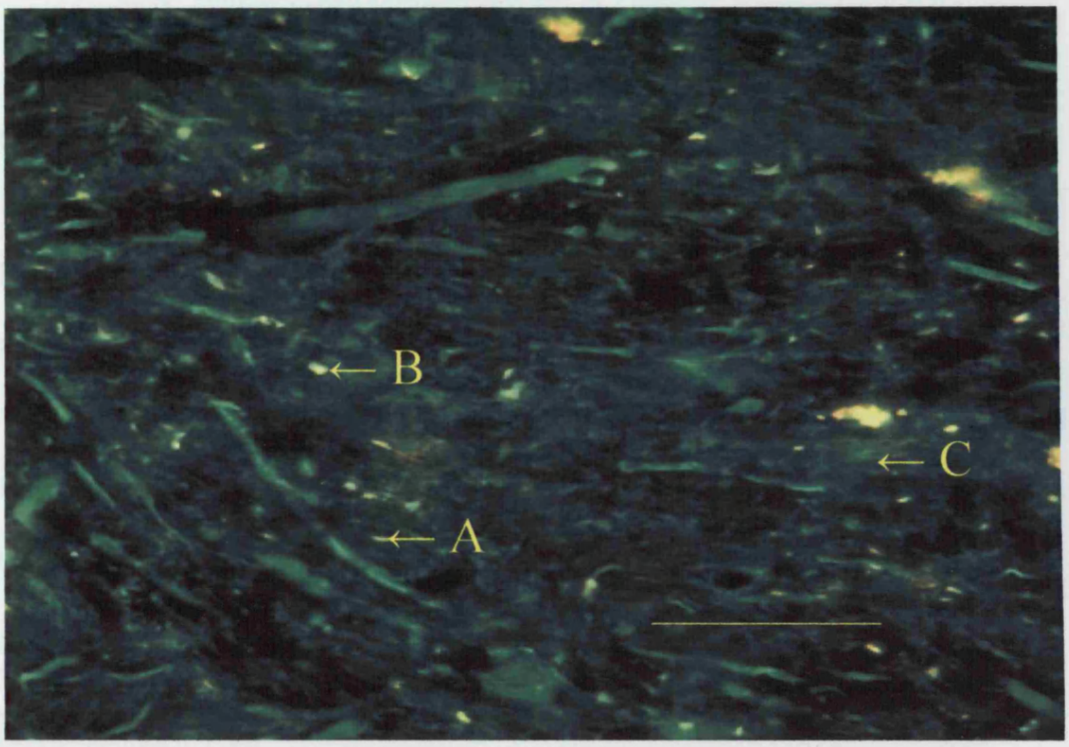


E: SC sample diluted 1/50 and tested against Human basal ganglia. Strong staining (arrow) of axons, mag x200

Key: Yellow bar 5µm

F: Persistent SC sample diluted 1/50 and tested against human basal ganglia. Staining of axons (arrows), mag x200

Figure 3-8 Detailed figure of a typical indirect immunofluorescence staining pattern in a patient with acute Sydenham's chorea using 1/50 diluted serum



A: Neuron specific staining; predominant in axons with sparing of the nucleus; B: Lipofuschin granules; C: Low background fluorescence (original magnification x200).
Key: Yellow bar 5µm

3.10 Indirect immunofluorescence of Putamen using 5 Sydenham's chorea samples

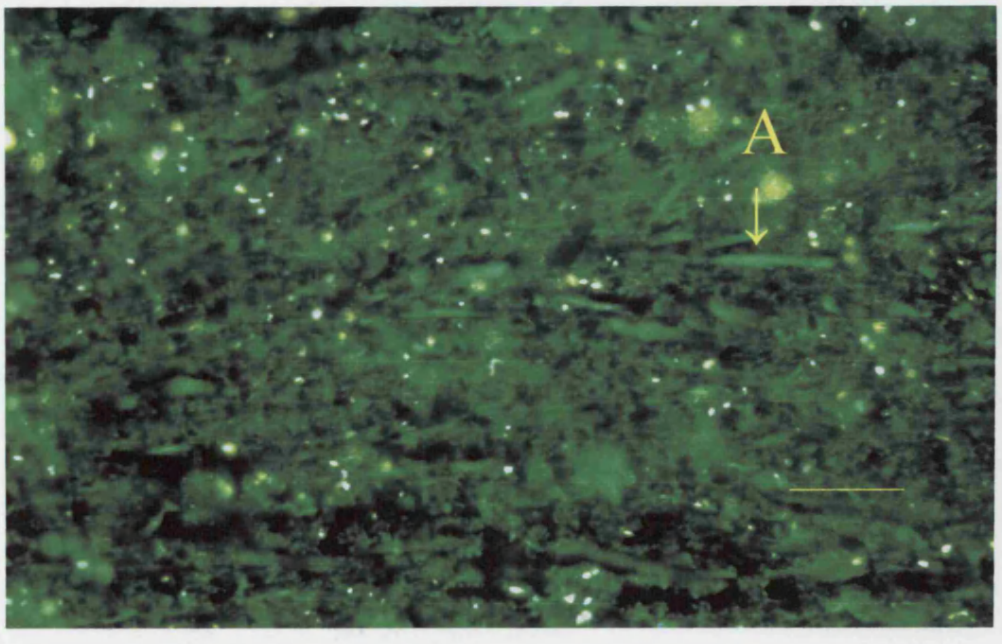
Introduction

To assess differences in caudate/putamen staining, 5 SC samples were tested at 1/25 and 1/50 against human putamen sections.

Results

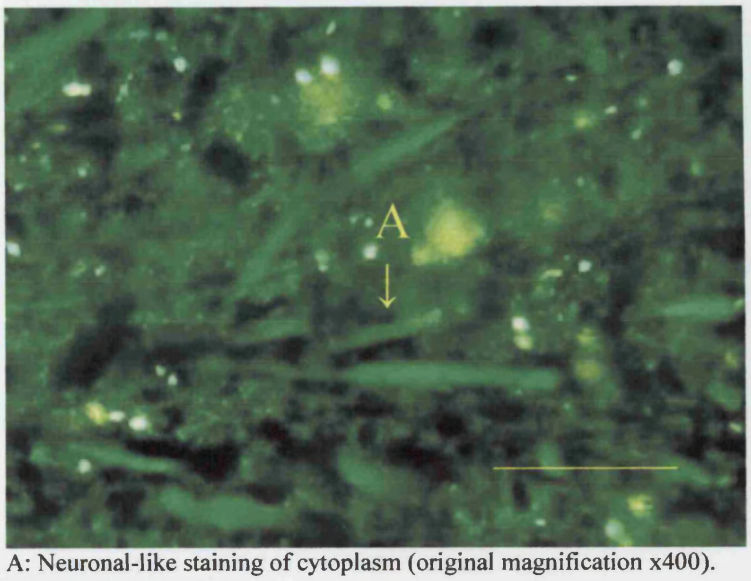
ABGA staining was only scantly present in putamen (Figure 3-9) and only at dilutions of 1/25, but appeared to be specific to the cytoplasm. High magnification appeared to show cytoplasmic and not membranous neuronal-like staining (Figure 3-10).

Figure 3-9 Typical Indirect Immunofluorescence staining pattern in the putamen from one patient with acute Sydenham's chorea using 1/25 diluted serum



A: Neuronal staining is present but positive neurones are sparser than seen in the caudate (original magnification x200). **Key: Yellow bar 5μm**

Figure 3-10 High power magnification of the same SC sample showing cytoplasmic staining of neuronal-like cell



A: Neuronal-like staining of cytoplasm (original magnification x400).
Key: Yellow bar 5μm

Conclusions

SC samples had an anti-neuronal like positive staining but only at 1/50 dilutions, this was stronger in caudate than putamen, the numbers of samples tested was small, n=20. Characterisation of neurones was crude, but, if glial cells were stained the numbers of positive stained cells would be far greater than that seen (Figure 3-9). As basal ganglia neurones express very similar receptors so an *in-situ* hybridisation method would be required to diffentiate neurones rather than just double staining with antibodies. The neurones appear to be large, 5μm in length.

However, using commercial antibodies to prove neuronal, rather than glial specificity of this low titre staining is required. Examining a larger cohort for sensitivity and specificity of IF is also required if ABGA are a diagnostic tool.

3.11 Anti-basal ganglia antibodies: neuronal specificity in human basal ganglia

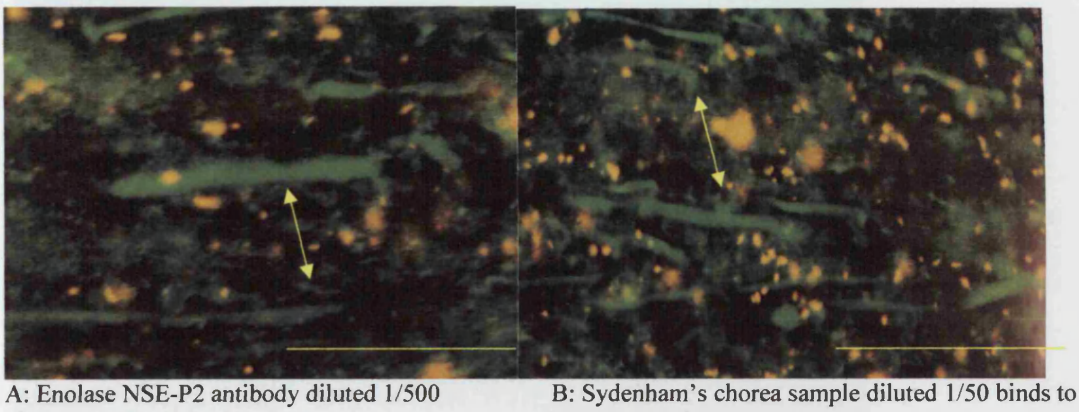
Introduction

The cells that stained with ABGA were also stained with a coomercial, neurone-specific enolase antibody. To assess the distribution of this staining, i.e. if staining is just cytoplasmic or there is any indication of membranous staining, human sections were tested. (Figure 3-11 and 12). Transverse sections were also tested as this was how Husby *et al* presented ABGA in their original paper (Husby *et al.*, 1976). Therefore an attempt was made to repeat this study and try and show if the cytoplasm of neurones were stained by IgG (Husby *et al.*, 1976).

Results

Commercial antibody against neurone specific enolase (NSE), which is a specific antibody against a protein found on and within neurones (Figures 3-11). Patients with SC had similar staining (Figure 3-7 and 3-8). Transverse section showed C-19 antibody stained neuronal cytoplasm (Figure 3-12). One patient with SC also had similar staining to the NSE antibody (Figure 3-13). There was no obvious membranous staining.

Figure 3-11 Longitudinal section of human basal ganglia stained with anti-enolase commercial antibody (C-19) diluted 1/500 to visualise axonal staining

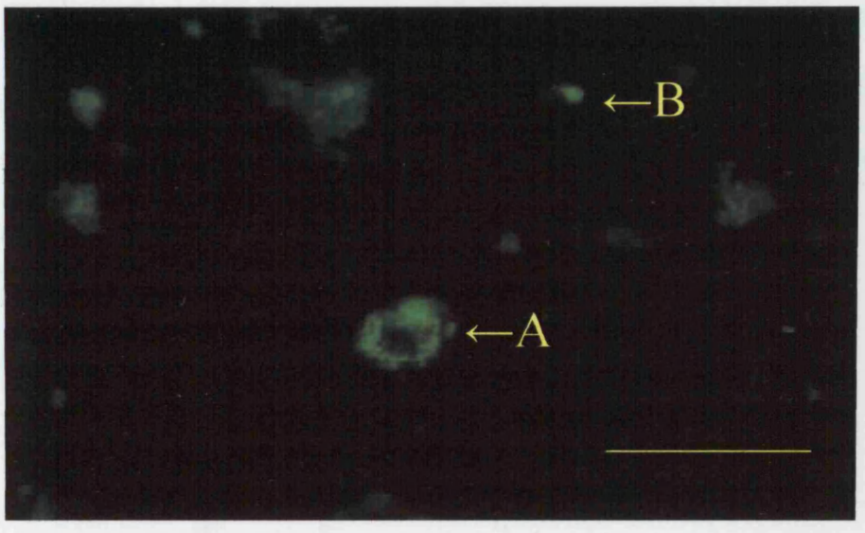


A: Enolase NSE-P2 antibody diluted 1/500 binds to axons (arrow).
Granules, mag x400

B: Sydenham's chorea sample diluted 1/50 binds to axons (arrow)
Ubiqutous lipofuschin granules
mag x400

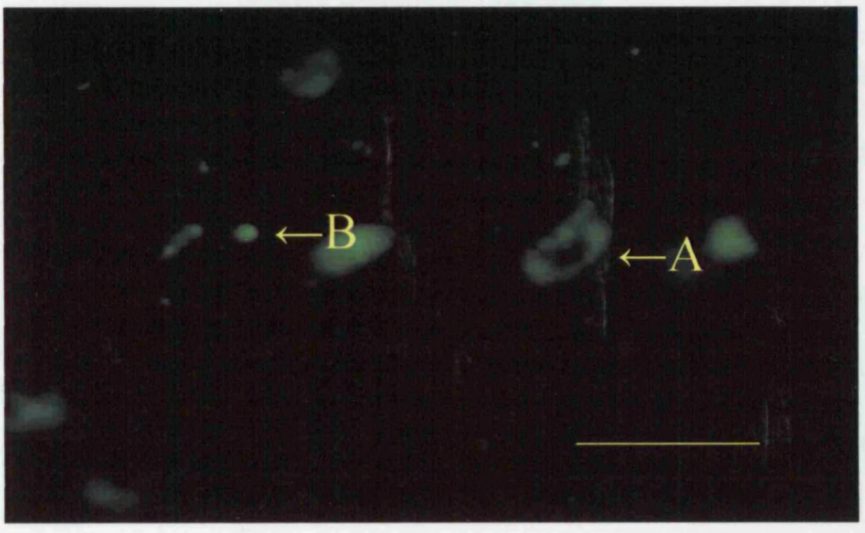
Key: Yellow bar=5μm

Figure 3-12Transverse section of human striatum stained with anti-enolase commercial antibody (C-19) diluted 1/500 to visualise neurones



A: Anti-enolase antibody staining of transverse section showing granular cytoplasmic staining of neurone;
B: Lipofuschin ganule (appears green due to filter used), (Original magnification x800).
Key: Yellow bar 5μm

Figure 3-13 Transverse section of human striatum stained with anti-basal ganglia positive antibody to visualise neurones



A: Sydenham's chorea anti-basal ganglia antibody staining of longitudinal section showing cytoplasmic staining of neurone; B: Lipofuschin granule (appears green due to filter used), (Original magnification x800). **Key: Yellow bar 5μm**

Conclusion

These results appear to show a axonal distribution of IgG binding from patients with SC but more samples should be tested. Commercial controls of ubiquitous neuronal proteins should also be tested such as neurofilaments, tubulin and 14-3-3. Live cell assays with primary striatal cultures are also necessary to prove this hypothesis.

3.12 Anti-basal ganglia antibodies in cerebrospinal fluid detected using indirect immunofluorescence

The volume of CSF from 2 acute SC patients was sufficient for ABGA testing, and this resulted in the same neuronal staining pattern as seen in the parallel serum samples. The ABGA positivity was the same as that found in the first SC cohort with cytoplasmic staining of medium-large neurones which were confined to neuronal tracts predominantly in the caudate head. However, the numbers tested are too small to draw any meaningful conclusions and SC patients are unlikely to undergo routine lumbar puncture.

3.13 False positive indirect immunofluorescence in a patient with chorea

One UK patient with a diagnosis of persistent SC was later diagnosed as having a vascular basal ganglia pathogenesis rather than SC. The IF pattern was consistent with that seen in SC and there was reactivity to several antigens on Western immunoblotting. The ABGA positive result may have been an example of a false positive result in a patient with suspected persistent SC, or the patient may have had a dual pathogenesis, having had clinically defined SC 2 years previously. In view of the specificity of ABGA the latter seems more likely.

3.14 Recrudescent (late recurrent) Sydenham's chorea

None of the 5 patients with recrudescent chorea was ABGA positive using IF, ELISA or Western immunoblotting. The mean ASOT level was 190 IU/mL (50-210 IU/mL). One patient (1/5, 20%) had a raised ASOT level of 210 IU/mL. The mean ant-DNAse B was 255 IU/mL (50-290IU/mL). No patient had a raised anti-DNAse B. One patient (1/5, 20%) was subsequently diagnosed with Coeliac disease and was placed on a gluten free diet. This improved the choreic symptoms but it is uncertain whether the improvement was a spontaneous improvement or related to the removal of gluten.

3.15 Ten Tourette's syndrome patients tested against basal ganglia

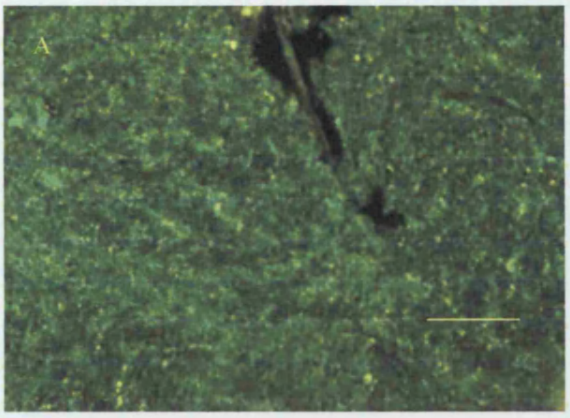
Introduction

IF was only performed in this study on 10 TS patients (5 paediatric and 5 adult) who were positive on Western immunoblotting. Ten, neurological disease controls were tested. All patients had the same binding pattern, with IgG binding to large basal ganglia neuronal-like cells.

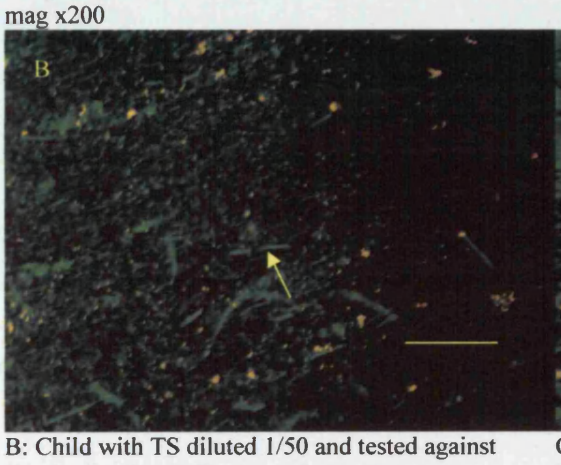
Results

IgG from all the TS patients studied had staining of axons with a similar pattern to SC (Figure 3-14 and Figure 3-15). None of the 10 controls tested reacted against any cellular component of the basal ganglia (Figure 3-14a).

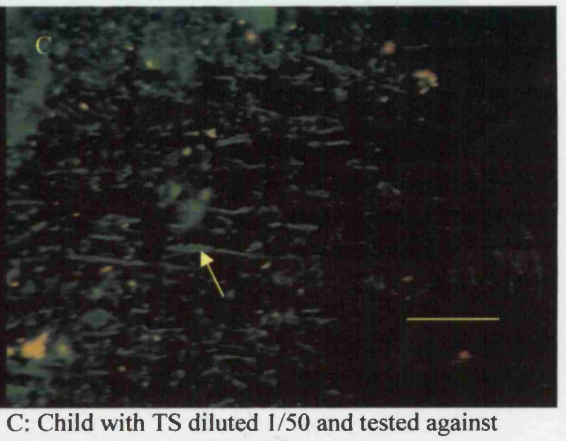
Figure 3-14 Indirect Immunofluorescence against human basal ganglia in Tourette's syndrome



A: Neurological disease control diluted 1/50 and tested against human Basal ganglia tissue. High background but no specific neuronal-like staining
mag x200



B: Child with TS diluted 1/50 and tested against Human basal ganglia. Axonal staining Present, mag x200
Key: Yellow bar 5µm

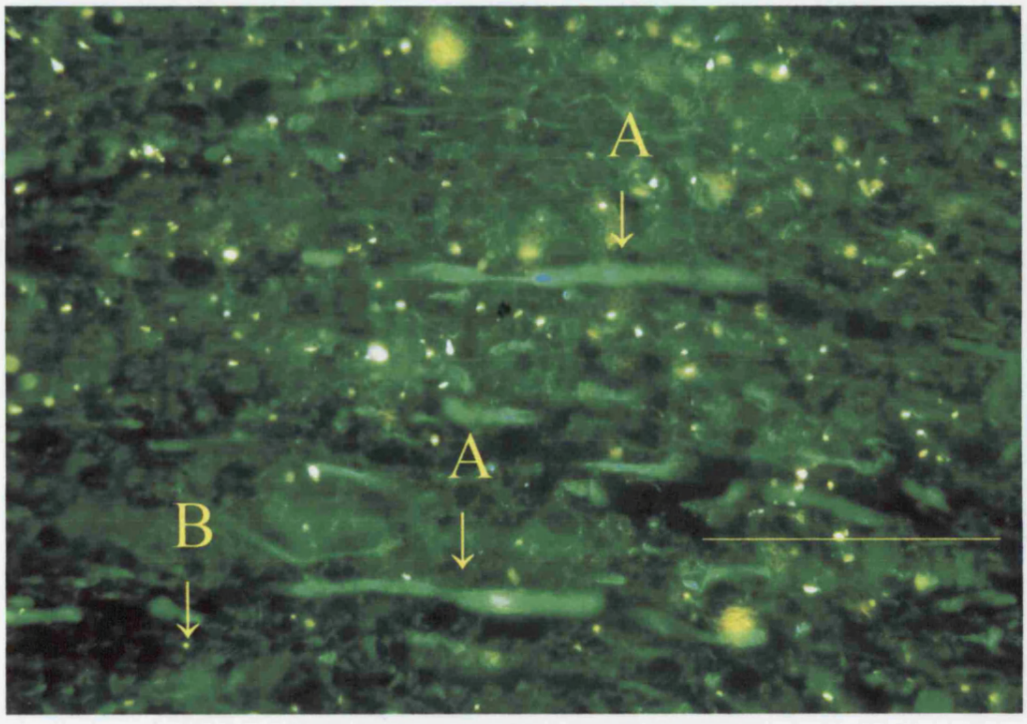


C: Child with TS diluted 1/50 and tested against Human basal ganglia. Axonal staining present showing cytoplasmic predominance at high Magnification, x400

Conclusions

IF was only used to investigate a small number of TS patients with positive streptococcal serology. A large histological study is required so this is pilot data only.

Figure 3-15 Detailed figure of indirect immunofluorescence of a sample from a patient with Tourette's syndrome tested at a 1/50 dilution against human caudate and putamen section



A: IgG staining of neuronal-like cells predominately localised to the cytoplasm; B: Lipofuschin granules; (Original magnification x400). **Key: Yellow bar 5µm**

3.16 Sixteen patients with Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections tested against human basal ganglia

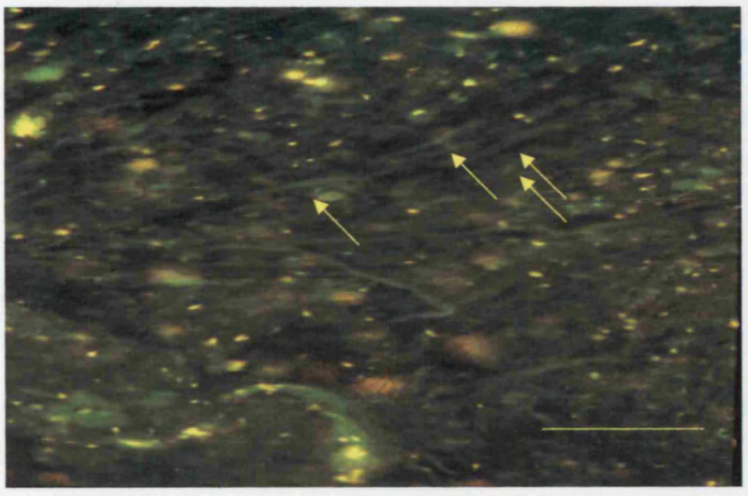
Introduction

Indirect immunofluorescence was performed in 16 patients with PANDAS and 10 with streptococcal infection controls

Results

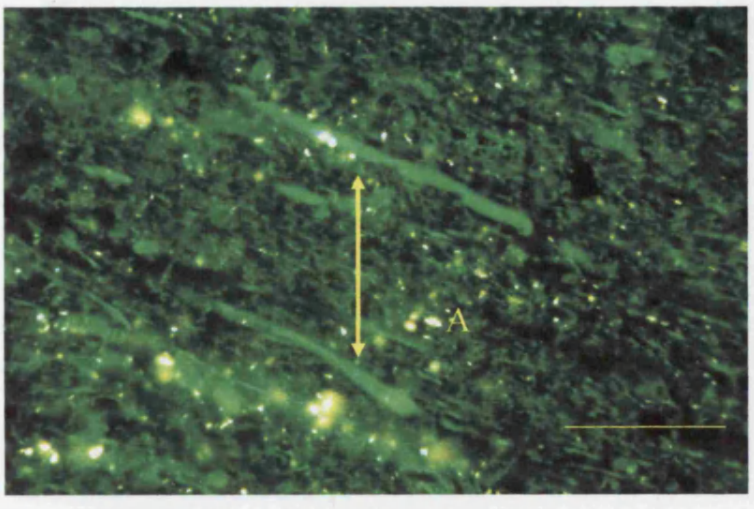
In the control group 1/10 streptococcal infection controls had similar binding to SC and TS at 1/50 (Figure 3-16), the remaining 9 were negative. The same IgG binding to neuronal-like cells was seen in 14/16 of PANDAS patients. The binding pattern appeared to be cytoplasmic and axonal if these cells are neurones (Figure 3-17).

Figure 3-16 Post-streptococcal nephritis control diluted 1/50 and tested against human basal ganglia showing neuronal-like binding



Axonal-like staining (arrows), mag x200, **Key: yellow bar 5µm**

Figure 3-17 1/50 dilution of sera from a UK PANDAS patient, tested with a section of human caudate and putamen tissue



A: IgG staining of the axons of large striatal neurones with the same pattern as that seen in the Brazilian SC patients. (Original magnification x400).
Key: Yellow bar 5µm

Conclusion

Whilst the similar staining in PANDAS to SC is interesting only a small number of samples were tested. This is pilot data only due to the small numbers of samples tested

3.17 The presence of other autoantibodies in Sydenham's chorea

Introduction

All SC patients and controls were tested for the presence of systemic autoantibodies using a general indirect immunofluorescence screen against rat liver, kidney and stomach tissue (The Binding site FS313).

Results

Few systemic autoantibodies (anti-nuclear antibodies etc) were detected in SC, RHF and controls (Table 3-4).

Conclusion

There was no difference in the number and percentage of positive systemic autoantibodies detected using IF in SC and controls.

Table 3-4 Other autoantibodies in Sydenham's chorea using indirect immunofluorescence

Autoantibody	Acute SC	Persistent SC	RHF	Normal controls
Nuclear antibodies	3/34 (10%)*	0/24 (0%)	3/20 (15%)	1/40 (3%)
Smooth muscle antibodies	2/34 (6%)	0/24 (0%)	3/20 (15%)	0/40 (0%)
Mitochondrial	0/34 (0%)	0/24 (0%)	0/20 (0%)	1/40 (3%)
Reticulin	0/34 (0%)	0/24 (0%)	0/20 (0%)	0/40 (0%)
Liver Kidney Microsomes	0/34 (0%)	0/24 (0%)	0/20 (0%)	0/40 (0%)
Ribosomal	0/34 (0%)	0/24 (0%)	0/20 (0%)	0/40 (0%)

P=n/s Fisher's exact test (2 tailed).* All 3 ANA positive patients were negative for dsDNA and extractable nuclear antigen autoantibodies.

Conclusions

Testing SC samples against rat brain sections did not result in any obvious staining of brain at a dilution of 1/500. There was only weak neuronal-like staining at 1/50 dilutions. The area of the brain with deep grey matter/basal ganglia like structure was identified with staining the section with a commercial monoclonal D2 receptor antibody. This stained a small portion of the section. There was no obvious staining of this area with SC samples compared to the rest of the brain. However, too few samples were tested and a more appropriate study would be at least 100 samples and 100 controls and to use histochemistry against whole rat brain with multiple monoclonal anti-neuronal antibodies to define cell populations.

As ABGA was originally reported using human brain (Husby *et al.*, 1976). Antigen localisation, presentation and presence might be dependant on species and may have accounted for the negative findings. Primate and human sections were also studied as previous published peer reviewed work of basal-ganglia antibodies used human tissue, so it appeared logical to at least attempt to replicate these frequently cited studies.

There was no staining of Purkinje cells with 1/50 diluted SC, TS or PANDAS samples in monkey sections. There was staining of neurone-like cells in the cerebellar granule layer of monkey brain. There was also neuronal-like staining in human basal ganglia. Commercial antibodies suggested this staining as neuronal rather than glial, although this is just a basic method. Techniques such as *in situ* hybridisation are required to characterise these neurones, this has not been done. Double staining sections with

antibodies against tubulin, neurofilaments and S100b could have been done to differentiate between neurones and glia. Further staining with commercial antibodies could not be done due to cost although more samples and controls must be investigated.

Human basal ganglia appeared to give the best staining of neuronal-like antibodies in SC, TS and PANDAS. Staining of neurones in other areas of the brain, notably the cerebellar granular layer means that these antibodies are not basal ganglia specific. However the antigens may be enriched in mammalian basal ganglia as the human sections gave a clear anti-neuronal staining pattern. The problem with this tissue source is high background fluorescence and problem of availability. Although controls were negative, presence of donor IgG might have caused false positive staining, so human tissue is not ideal for study.

Further studies are required to investigate the specificity of anti-neuronal antibodies in SC. Additional techniques could include immunohistochemistry and live cell assays using primary striatal cultures. One potential method is using cultured cerebellar granule cells which appeared to be bound by IgG from SC patients according to Dale *et al* (Dale *et al.*, 2006). An ELISA screening test for ABGA was developed for screening multiple samples to identify samples for proteomics and putative antigen identification.

4 Results of the Enzyme linked immunoabsorbant assay

4.1 Anti-basal ganglia antibodies in Sydenham's chorea detected by ELISA; method development for a screening assay

Introduction

Indirect immunofluorescence appeared to suggest cytoplasmic, rather than membranous distribution of weak (1/50) IgG binding from patients with Sydenham's chorea, PANDAS and a small group of patients with Tourette's. This may suggest that the antigen(s) responsible for this staining are cytosolic and therefore possible soluble. It was hypothesised that a soluble fraction obtained from basal ganglia tissue might be an ideal antigen for an ELISA to detect these antibodies in large numbers of samples. The ELISA technique has the advantage of cheaper cost and availability of tissue from animal sources if the assay was discriminatory between SC, PANDAS and controls.

A box-and-whisker plot, incorporating a scatter plot of individual data points is used to show all ELISA data. The bar represents the median, rather than mean absorbance and the whisker is the range of results.

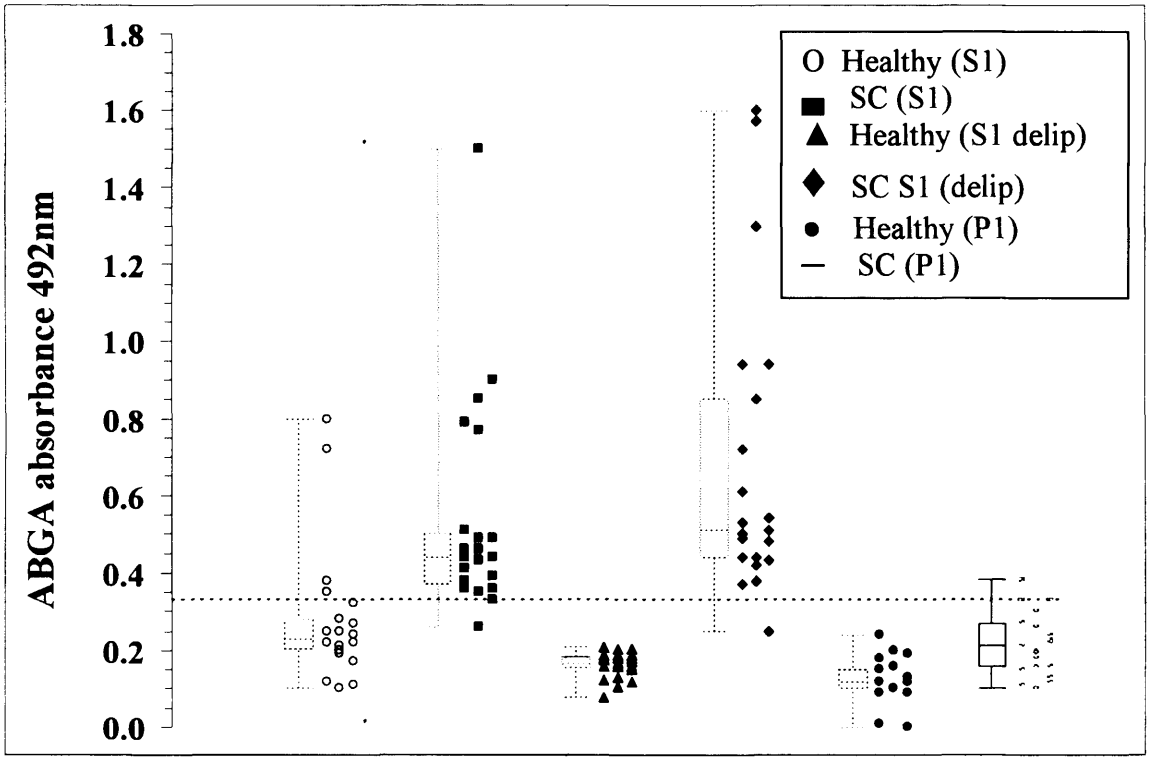
4.2 Comparison of basal ganglia preparations for ELISA; whole homogenate, de-lipidated soluble and insoluble fractions

The influence of different antigen preparations on the ELISA results was investigated. Duplicate microtitre plates were prepared using the same concentration of either (1) soluble basal ganglia homogenate S1 (2) The S1 fraction treated to remove excess lipid (delipidated); (3) Insoluble fraction P1 (solublised with 6M urea), (Figure 4.1). Each plate was incubated with the same samples (20 acute SC and 20 normal controls).

Results

The soluble whole homogenate ELISA had high blank wells (mean 0.1 absorbance, SD 0.08). There were also high absorbances (>0.35) in four normal controls (Figure 4-1). The presence of myelin-lipids in the homogenate could have caused these results and removal of lipids reduced these raised results in controls (Figure 4-1).

Figure 4-1 Results from twenty Sydenham's chorea samples and twenty normal controls diluted 1/100 showing ABGA ELISA absorbances plotted against different antigen preparations with error bars showing the range of results



S1=soluble human basal ganglia fraction; S1 delip= S1fraction delipidated; P1 fraction=solublised remaining pellet after initial homogenisation and removal of soluble proteins. All samples were tested in duplicate.

The insoluble fraction P1 did not produce any differences in SC patients compared to controls (p=n/s in all instances). The S1 fraction was treated to remove lipid and investigated for use as an ELISA antigen to detect anti-brain antibodies in SC, PANDAS, TS and controls.

4.3 The optimum fraction for performing an ELISA to detect anti-basal ganglia antibodies in 20 Sydenham's chorea and 20 normal controls

The results showed that the best signal-to-noise result of the 20 SC samples compared to controls and lowest blanks, was from the soluble fraction of basal ganglia S1 (caudate and putamen), prepared using T-per but, treated with Di-iso-propyl-ether (BDH) to remove excess lipid (Figure 4.1).

Results

Reagent blanks of S1 antigen typically had a mean absorbance of 0.05 (SD 0.03). However, there was no significant difference (p=n/s) in ABGA results when comparing mean SC results from the S1 fraction (mean absorbance 0.78, SD 0.06) compared to the de-lipidated S1 fraction (mean absorbance 0.64, SD 0.09), (Figure 4.1). Therefore the de-lipidated S1 fraction was used for all ELISA.

Conclusion

The potential auto-antigens that patients with SC react to are soluble proteins rather than lipids, or membrane proteins and are cytosolic. Therefore ABGA are probably just a marker of disease rather than functional on the basis of these results with the IF findings.

4.4 Optimisation of ELISA method using S1 soluble fraction of human basal ganglia

Introduction

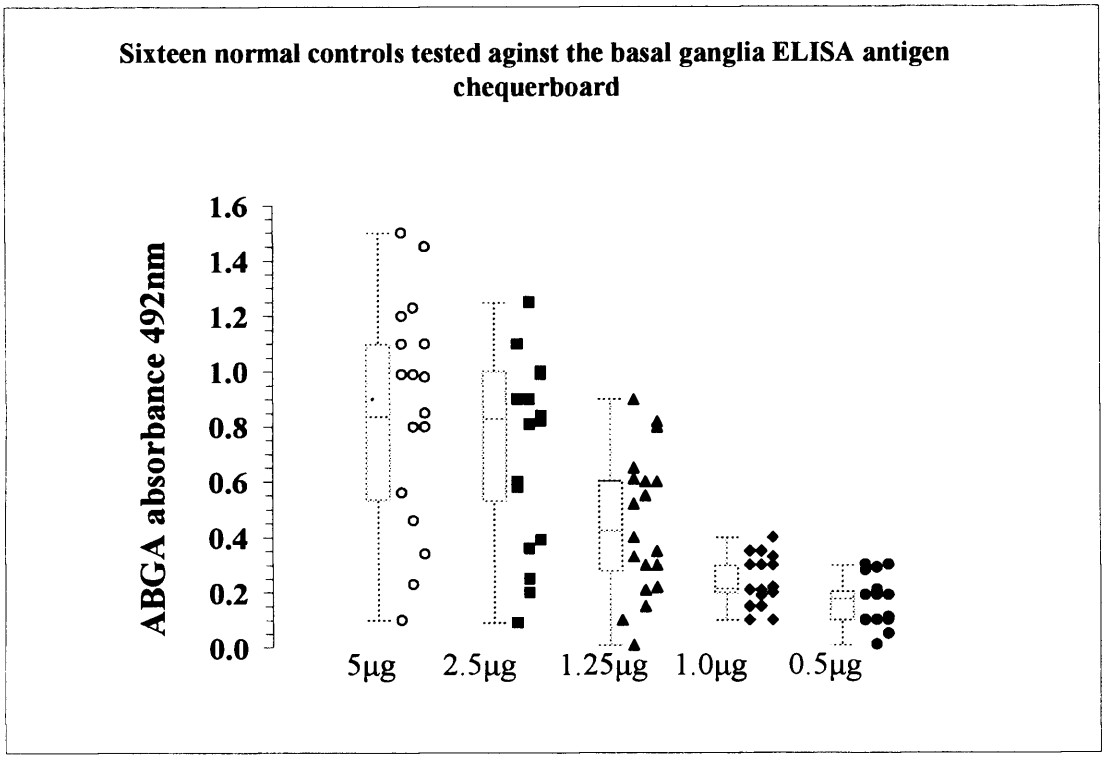
The antigen concentration for the ABGA ELISA method (standard serum dilutions and dilution of the detecting secondary antibody) was obtained from the chequerboard of dilutions using 16 SC and 16 normal controls (Figure 4.2). The optimum components of the ELISA:

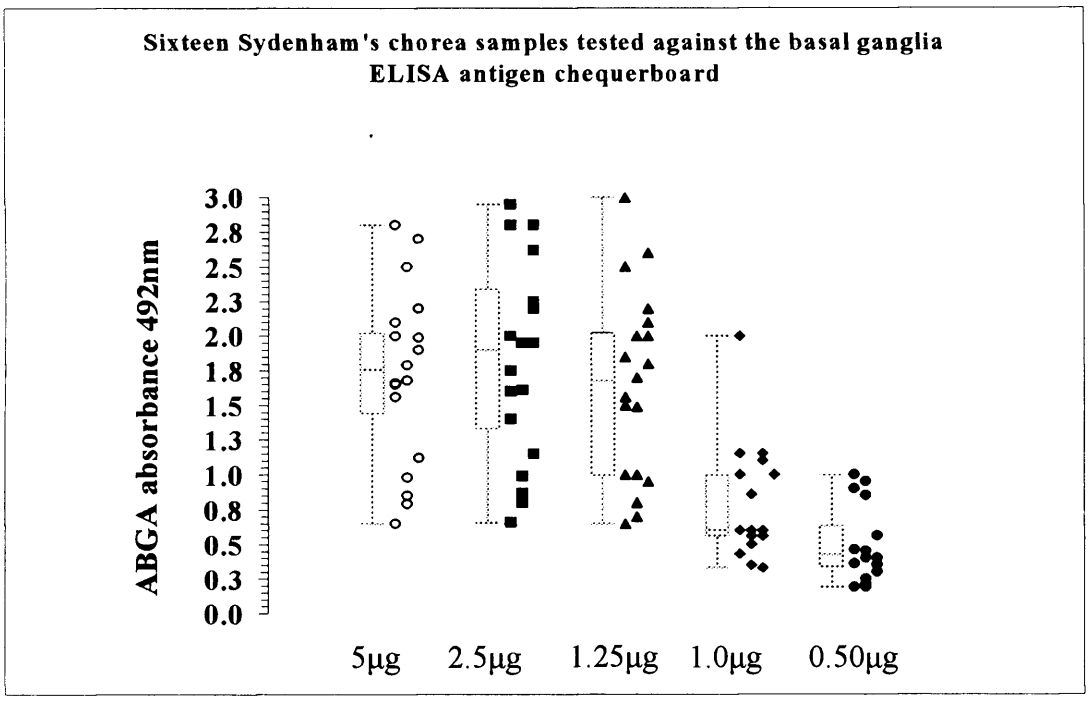
- Concentration of antigen which gave the best signal-to-noise ratio with low absorbance of the reagent blanks (<0.10).
- Serum and secondary antibody dilutions that gave the best signal-to-noise ratio
- The ELISA was designed to give ideal discrimination between absorbances from IF negative and positive samples so that it could be used as a screening assay.

Results

The ELISA antigen concentration used was 1μg/mL of human basal ganglia soluble fraction which was delipidated (Figure 4-2). However, the mid-point of the concentration chquerboard was 1.25μg/mL (Figure 4-2) which could also be used. Using 1μg/mL the mean absorbance of normal controls was under 0.5 which is the cut-off for a screening ELISA used routinely by the Neuroimmunology department, UCL NHS trust, CPA accredited, to detect anti-ganglioside antibodies in GBS. This concentration appeared to allow discrimination between SC patients which had a mean absorbance in these 16 patients of over 0.5 and the normal controls (Figure 4-2).

Figure 4-2 Results of sixteen normal controls and acute Sydenham's chorea samples diluted 1/100 and tested against soluble S1 basal ganglia ELISA antigen concentration chequerboard





4.5 Optimum serum dilution for ELISA

A series of serum doubling dilutions of 16 SC samples resulted in a 1/300 dilution of serum giving the optimum signal-to-noise ratio between controls and positive samples (Figure 4.3). Additionally, a typical double dilution of 1 SC samples is presented in Figure 4.4. This was also a similar dilution (1/250) used by Singer *et al's* group for their basal ganglia ELISA method (Singer *et al.*, 1998 and 2003).

Figure 4-3 Serial serum dilutions to ascertain optimum dilution for anti-basal ganglia ELISA using sixteen Sydenham's chorea samples

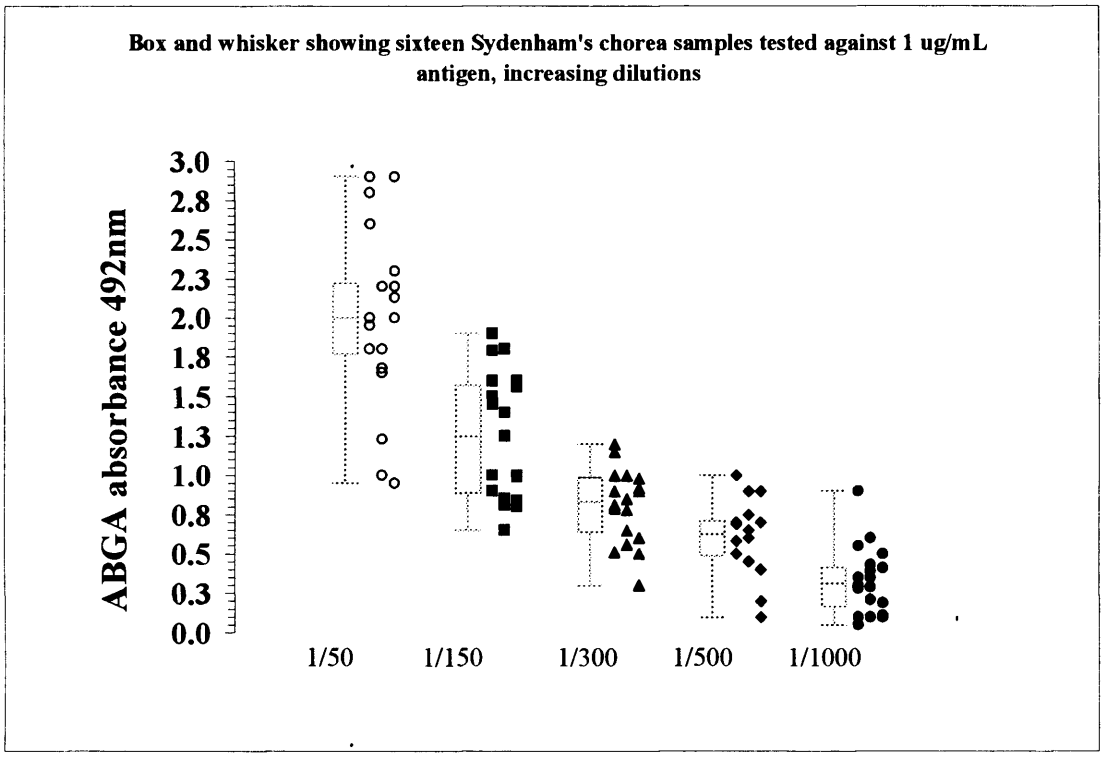
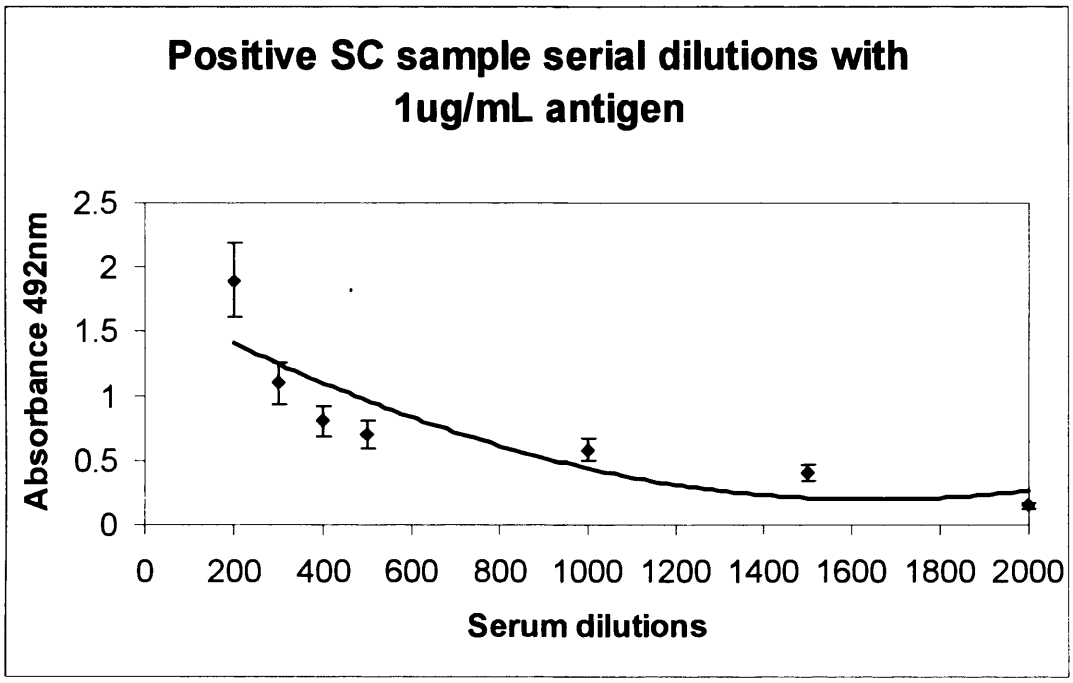


Figure 4-4 Titration result from 1 patient with error bars showing percentage difference in absorbances after testing 3 times



4.6 Optimum dilution of secondary anti-Human IgG HRP conjugated antibody

The optimum dilution of the secondary detector antibody was obtained through following guidance of the manufacturer for antibody (Dako). A dilution of 1/2000 of the secondary antibody was used.

4.7 Intra-assay variation of the basal ganglia ELISA

Introduction

Three SC samples and three normal controls were tested on the same plate using 1 μg/mL of soluble S1 fraction and 1/300 dilution of serum to assess intra-assay variation of the ELISA.

Results

The results are summarised in Table 4-1.

Table 4-1 Summary of the results of intra-assay variation for the basal ganglia ELISA

Group	Mean absorbance	SD	Standard error
Acute SC	0.63	0.14	0.04
	0.55	0.14	0.05
	0.68	0.11	0.03
Normal	0.19	0.06	0.02
controls	0.17	0.08	0.03
	0.25	0.06	0.02

4.8 Inter-assay variation of the basal ganglia antibody ELISA

One SC sample and 1 normal control were tested in duplicate wells on 10 different plates to assess inter-assay variation (Figure 4.5). The statistics revealed that the assay was relatively reproducible (Table 4-2).

Figure 4-5 Low and high positive ELISA sera tested on 10 separate ELISA plates to assess inter-assay variation of the ELISA

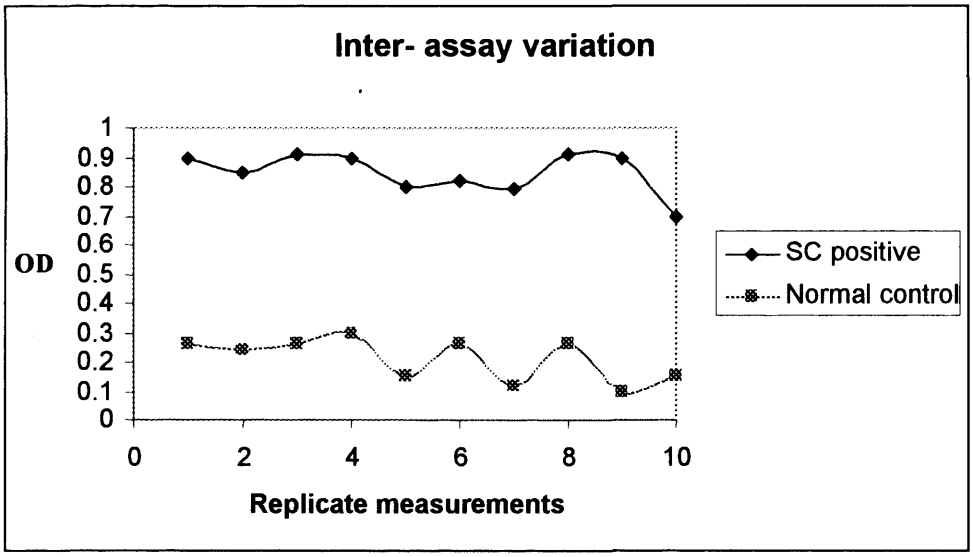


Table 4-2 Statistical analysis of inter-assay variation

Group	Mean absorbance	SD	CV	Standard error
Acute SC	0.85	0.07	0.08	0.02
Normal control	0.21	0.07	0.34	0.02

4.9 Summary of how the antigen was prepared for the ELISA used in all the following results

Defrost caudate/putamen block at room temperature, weigh and cut into small pieces with a sterile scalpel. Transfer into homogenisation tube, add protease inhibitor cocktail (100 µl per 2g tissue), (Sigma chemicals). Add T-per, tissue extraction reagent (Peirce), (1mL per gram of tissue). Place tube in wet ice and homogenise tissue until there are no clear precipitates. Transfer into a fresh tube and centrifuge at 10,000rpm for 15 minutes. Collect supernatant, mix with 25% volumes of di-iso-propyl-ether, centrifuge, 10,000rpm for 15 minutes. Using a spinal needle, or long glass pipette, collect the protein fraction from the solvent/protein phase and store in a fresh tube. All the ABGA ELISA results were obtained using 1µg/mL of basal ganglia homogenate and all samples were tested in duplicate at 1/300 dilution of serum. Measure total protein; adjust concentration to 1µg/mL in 0.025M carbonate buffer. Pipette 100 µl into duplicate well of a Nunc, maxisorb microtitre plate, cover with film and leave at +4°C overnight (12 hours). Rinse with PBS and proceed with ELISA run.

4.10 IgG levels in 30 Sydenham's chorea samples and 50 controls

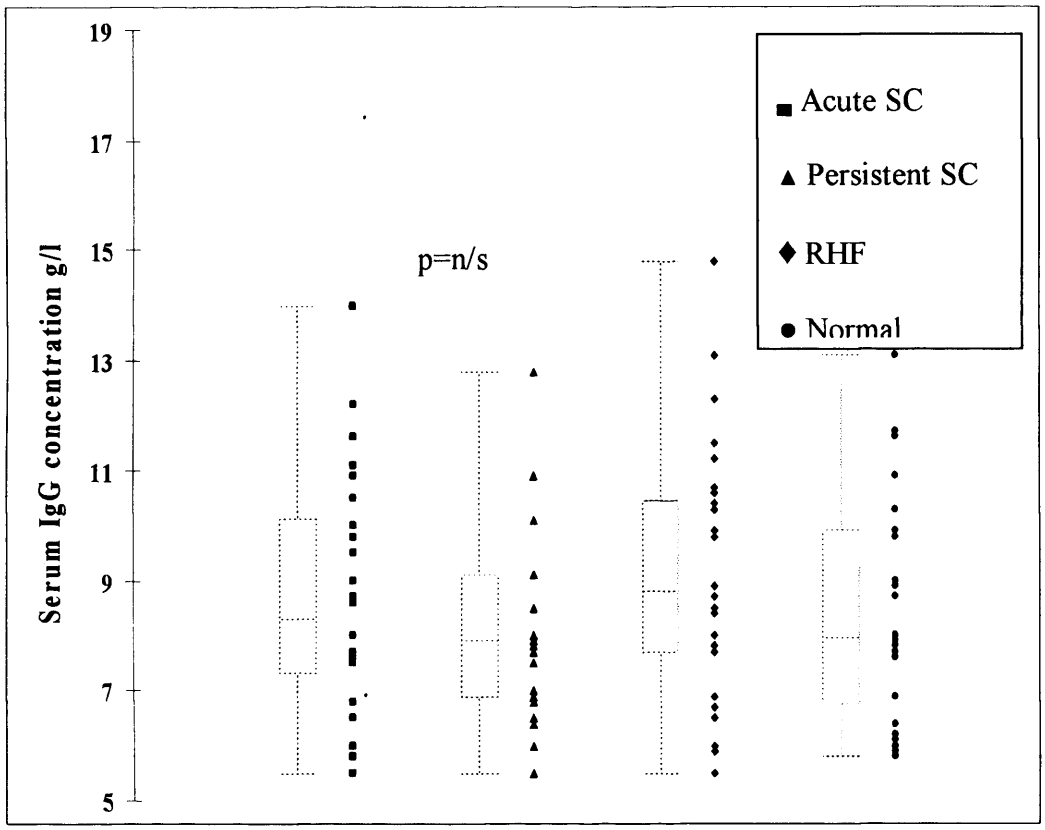
Introduction

To assess non-specific binding, IgG concentration was measured. However, the normal ranges of IgG are dependant on age and will influence the importance of these results. Therefore individual patients and controls will naturally have higher of lower IgG levels than older or younger children. This is a potential source of inaccuracy in the ELISA method and is a major critism of this assay. A better method would be to dilute all the samples to the same concentration of IgG but this was not practical for large numbers of samples and expensive.

Results

There appears to be no significant differences in mean IgG results between SC and controls (Figure 4-6). No variation in IgG concentration in age ranges was taken into account.

Figure 4-6 Serum IgG levels in 30 acute, 20 persistent Sydenham's chorea patients and 30 rheumatic fever and 20 normal controls measured by standard rate nephelemetry



p=n/s; Wilcoxon rank-sum test (Mann-Whitney)

4.11 Analysis of the basal ganglia ELISA results of 60 controls and 58 Sydenham's chorea samples

Introduction

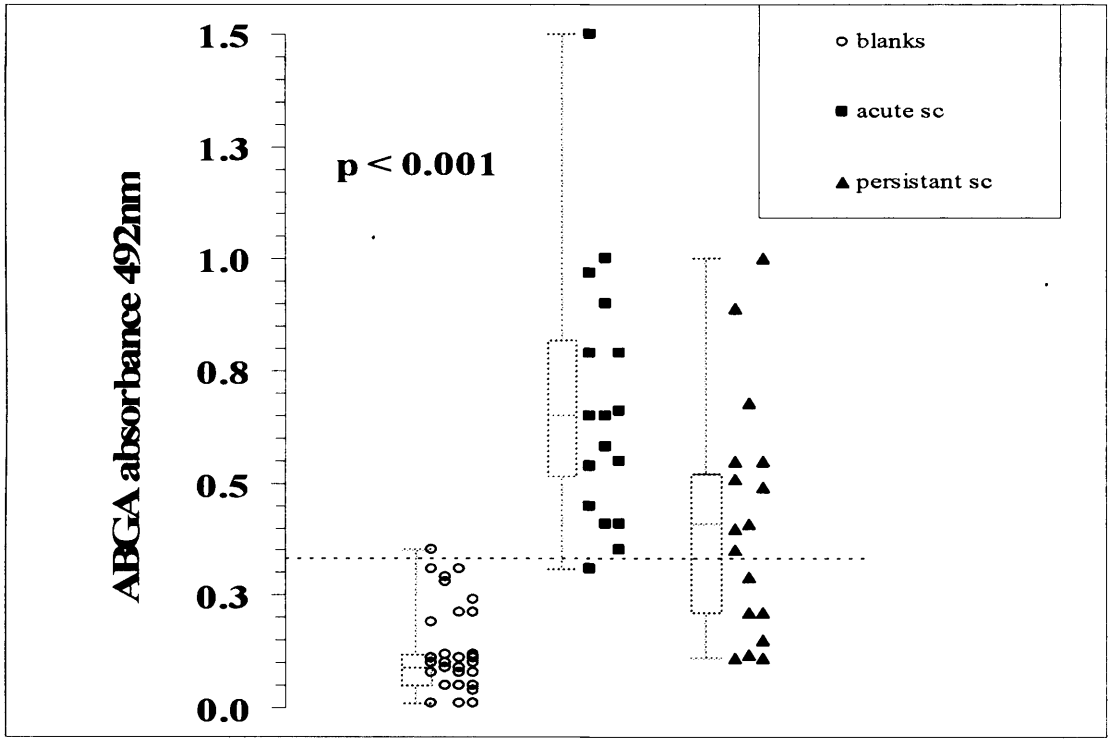
Using the optimum basal ganglia ELISA method 60 controls and 58 SC samples (Table 4-3) were tested 1/300 against ELISA of soluble S1 fraction.

Results

The mean absorbance in SC was increased compared to normal controls (p=<0.001) and patients with RHF (p=<0.001), (Wilcoxon rank-sum test), (Figure 4-7, Figure 4-8 and Table 4-3). There was no significant binding of IgG to just the microtitre plate, a method suggested by examiners (Figure 4-7 and Figure 4-8).

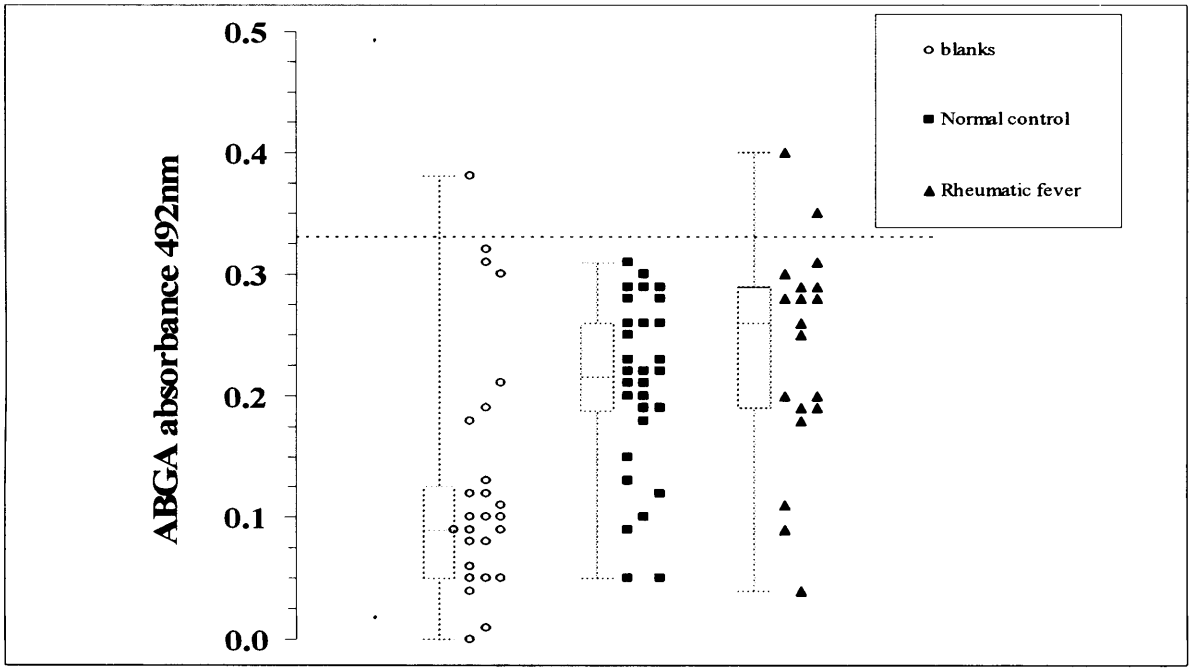
Subgroup analysis showed patients with acute SC had a higher mean ABGA absorbance than patients with persistent SC (p=0.03) or controls (p=<0.001). Patients with persistent SC also had significantly higher mean ABGA levels than normal (p=0.002) and RHF controls (p=0.003), (Figure 4-7, Figure 4-8 and Table 4-3).

Figure 4-7 Box and whisker plot of basal ganglia antibody ELISA absorbances of twenty acute and persistent Sydenham's chorea samples diluted 1/300 with individual blanks (no antigen) for each sample



p=<0.001 refers to acute SC versus blanks and versus persistent SC; Wilcoxon rank-sum test (Mann-Whitney). There was a significant increase in ABGA ELISA in acute SC compared to persistent SC

Figure 4-8 Box and whisker plot of basal ganglia antibody ELISA absorbances of twenty Rheumatic fever and forty normal controls samples diluted 1/300 with individual blanks (no antigen) for each sample



p=n/s; Wilcoxon rank-sum test (Mann-Whitney). There was no significant difference between Rheumatic fever and normal controls. The mean absorbance of blank wells (no antigen) was 0.127. There were 14/40 patients who had blank wells above the mean blank although only 1 patients had a blank result above a statistical cut off value 0.33 absorbance units.

Table 4-3 Summary of ELISA results of Sydenham's chorea patients

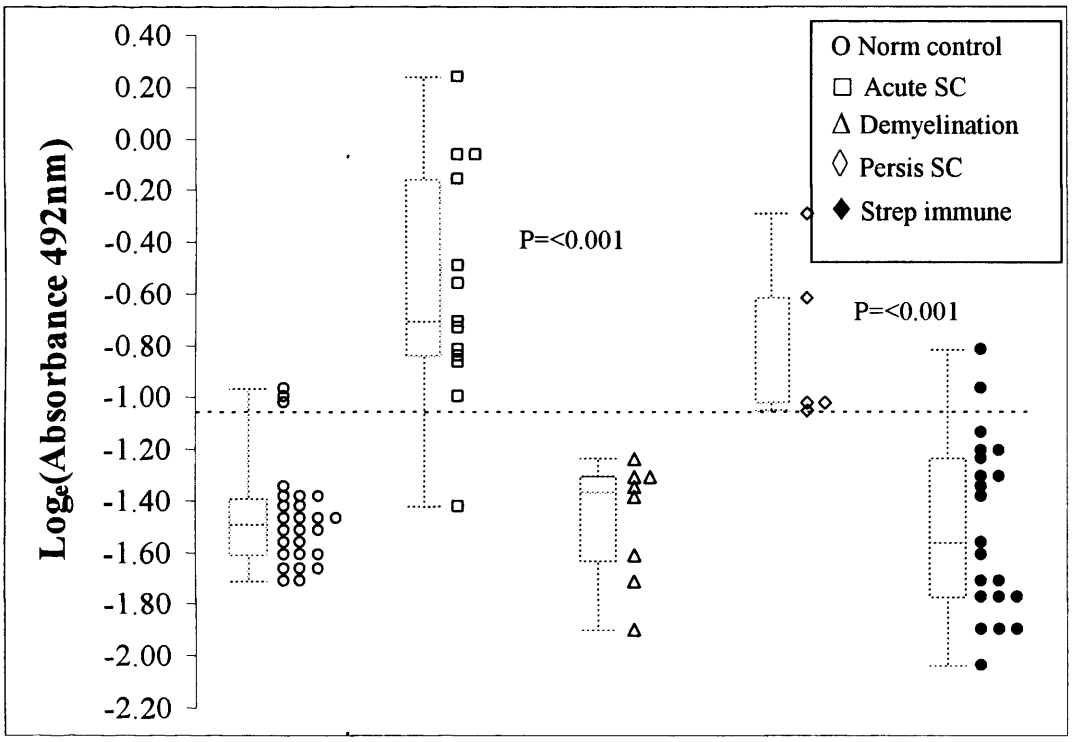
Group	Number	Mean absorbance at 492nm	SD
Acute SC	34	0.62*	0.2
Persistent SC	24	0.40**	0.15
Rheumatic fever	20	0.23	0.11
Normal controls	40	0.21	0.06

*p=<0.01, **p=0.03, (versus normal and rheumatic fever controls). Wilcoxon rank-sum test (Mann-Whitney).

4.11.1 Basal ganglia ELISA results using transformed data

Normalising the absorbance data, using a simple logarithmic transformation resulted in acute and persistent SC ELISA results significantly elevated compared to a range of normal, streptococcal and neurological disease controls (Figure 4-9). This confirms that an anti-basal ganglia screening ELISA detected significant elevations in IgG binding in Sydenham's chorea compared to a range of normal and disease controls.

Figure 4-9 Log transformed plot of anti-basal ganglia antibody ELISA in 20 acute, 6 persistent Sydenham's chorea patients and 30 normal, 10 demyelinating neurological and 25 streptococcal immune mediated disease controls.



p=<0.001; Wilcoxon rank-sum test (Mann-Whitney), Acute and persistent SC versus controls.

4.12 Basal ganglia ELISA utilising an upper limit of normal cut-off value

Introduction

The number and percentage of individuals in each group with ELISA results above normal was calculated. To assess raised ABGA ELISA results a cut-off value for normal limits was calculated, (mean of controls plus 2 standard deviations, 95% percentile).

Results

This produced a cut-off value of 0.33 absorbance units. A higher cut-off value, (control mean plus 3 standard deviations, 99% percentile) was also calculated. This produced a cut-off value of 0.39 absorbance units. Using these cut-off values the number and percentage of raised ABGA ELISA results in each group was calculated (Table 4-4). A cut-off value of 0.33 was found to give the best ABGA positive discrimination of SC patients from controls.

4.12.1 Positivity of Sydenham's chorea patients compared to ELISA cut-off

Using the cut-off value (0.33 absorbance units), 91% (31/34) of the acute SC group, 58% (14/24) of the persistent SC group, and 78% (45/58) of the entire SC cohort, 10% (2/20) of RHF and 0% (0/40) of normal controls had an elevated ABGA (Table 4-4). Cerebrospinal fluid could not be tested due to insufficient sample volume.

Table 4-4 Anti-basal ganglia antibodies in Sydenham's chorea using ELISA cut-off

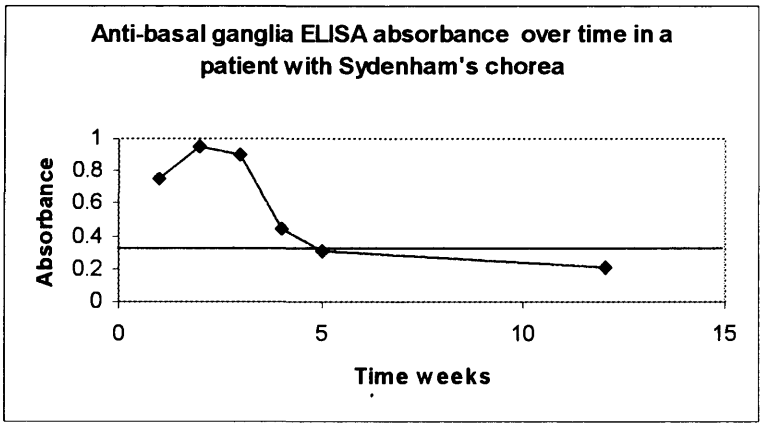
Group	Number >0.33 (Percentage)	Number >0.39 (Percentage)
Acute SC	31/34 (91%)**	18/34 (53%)*
Persistent SC	14/24 (58%)*	8/24 (33%)*
Rheumatic fever	2/20 (10%)	1/20 (5%)
Normal controls	0/40 (0%)	0/40 (0%)

*p=<0.01; ** p=<0.001, Fisher's exact test (2 tailed), Acute and persistent SC versus controls.

4.12.2 Anti-basal ganglia antibodies in a patient with Sydenham's chorea: ELISA results over time

One patient with a diagnosis of acute Sydenham's chorea was admitted to hospital and ABGA measured at 1 week intervals for 3 weeks. Two further samples were tested in the convalescent phase. The level of ABGA measured by ELISA was highest at week 2 when the chorea was at its worse (Figure 4-10). The level of ELISA absorbance fell over time and appeared to mirror that of the clinical recovery (personal correspondence from Dr Russell Dale, Great Ormond Street Hospital).

Figure 4-10 Anti-basal ganglia antibodies in one patient with acute Sydenham's chorea during acute disease and clinical recovery



4.13 Anti-basal ganglia antibodies: ELISA results in Sydenham's chorea patients and controls from the UK

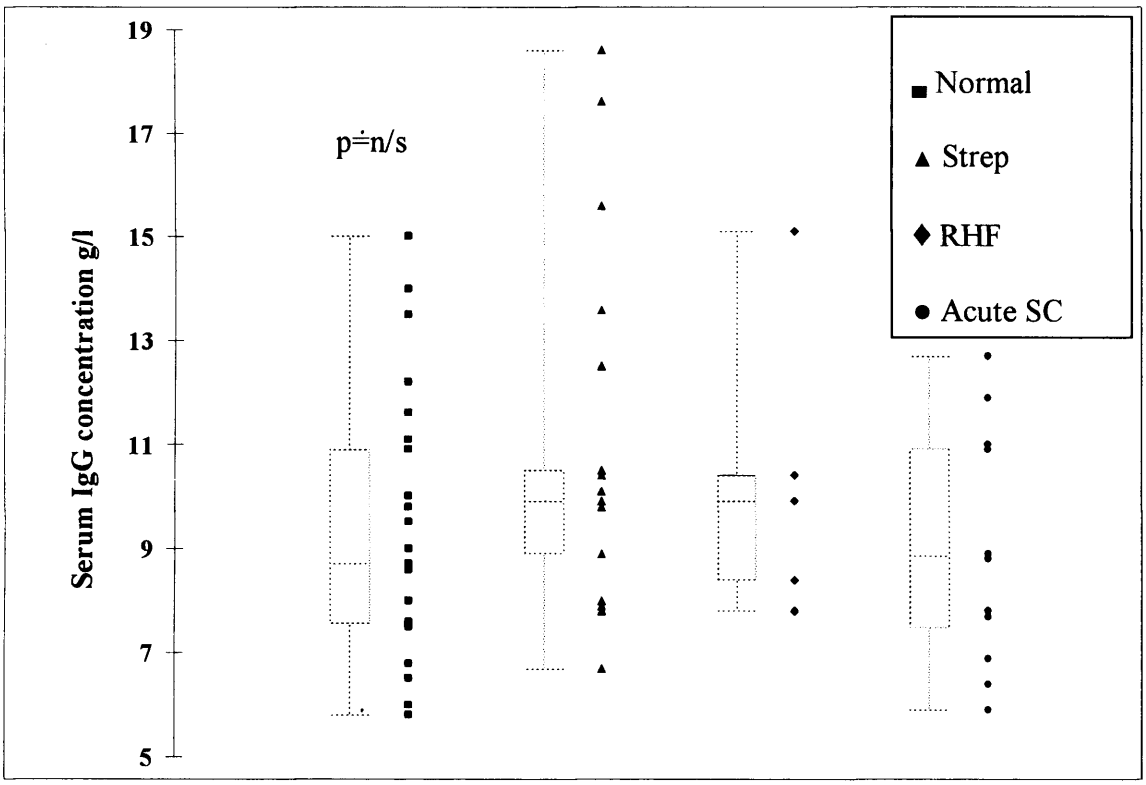
Introduction

Where possible serum was analysed for IgG concentration

Results

There was no difference in serum IgG between SC and controls (Figure 4-11) and the mean ABGA ELISA results were similar between all controls groups.

Figure 4-11 Serum IgG levels in 12 UK patients with acute SC, 5 with persistent SC compared to 20 UK development delay controls and 15 Streptococcal infection controls



p=n/s; Wilcoxon rank-sum test (Mann-Whitney).

Results of the basal ganglia ELISA

In the acute SC group the mean ABGA ELISA was raised compared to any control groups (P=<0.01 in all comparisons), (Wilcoxon rank-sum test). In the persistent SC group the mean ABGA ELISA was not different from any control and was lower than the acute SC group (P=<0.001), (Table 4-5). In the acute SC group, as in the Brazilian SC cohorts, ABGA was raised (>0.33) in 83% of patients (10/12) unlike any control group (P=<0.001 in all instances). The mean ABGA ELISA in the persistent SC group was not different from controls although the number tested was small. The number of persistent SC patients with ELISA results above the cut-off (>0.33) was higher (p=<0.001) than controls though 33% (1/3) and consistent with the findings from the Brazilian SC cohort (Tables 4-4 and 4-5). In the control groups 0-21% of the UK streptococcal-associated autoimmune disease controls had raised ELISA results (>0.33) which was higher than the Brazilian normal controls or the UK developmental delay controls (Table 4-5).

Table 4-5 Summary of Sydenham's chorea anti-basal ganglia antibody results (UK)

Samples	Number (sex M:F)	ELISA ABGA mean (Normal <0.33 absorbance units)	P value
Developmental delay controls	30 (15/15)	0.210 (0/30, 0%)	P=<0.0001
Post-streptococcal arthritis	15 (8/7)	0.231 (1/15, 6%)	P=<0.0001
Post-streptococcal glomerulonephritis	14 (5/9)	0.261 (3/14, 21%)	P=0.0048
Post-streptococcal vasculitides	15 (5/10)	0.201 (0/15, 0%)	P=<0.0001
Rheumatic fever	5 (1/4)	0.305 (0/5, 0%)	P=<0.0001
Acute Sydenham's chorea	12 (2/10)	0.565 (10/12, 83%)	-
Persistent Sydenham's chorea	3 (1/2)	0.286 (1/3, 33%)	-

P value; Fisher's exact test (2 tailed), Acute SC versus controls.

4.14 Anti-basal ganglia antibodies detected by ELISA in patients with PANDAS

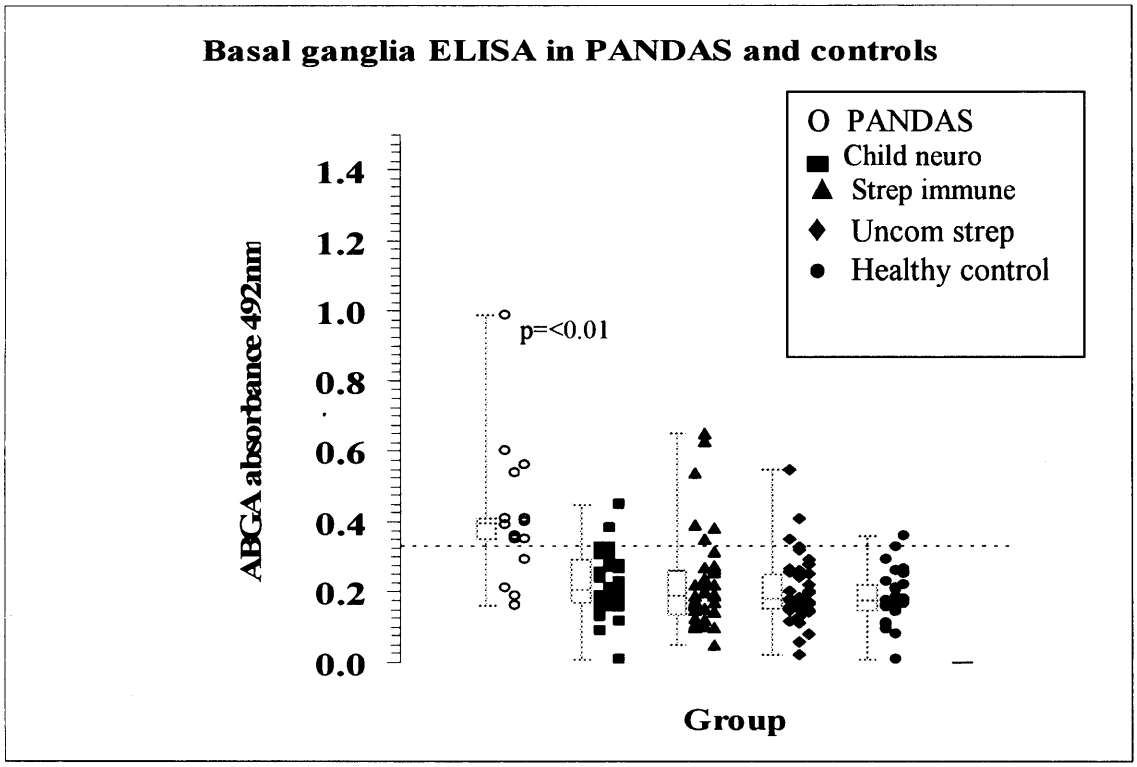
Introduction

Serum IgG was not tested routinely as there was no ethical approval for this test which is a recognised weakness and criticism of this study.

Results of basal ganglia ELISA

The mean ABGA ELISA absorbance was elevated in the PANDAS group compared to the neurological, streptococcal, autoimmune and normal controls (<0.001 in all comparisons), (Wilcoxon rank-sum test), (Figure 4-12). However, there was 47/220 (21%) of controls above the cut-off value for this ELISA so ELISA had a sensitivity of 75% (12/16 PANDAS positive) and specificity of 79% (47/220 positive control groups).

Figure 4-12 Box and whisker plot of basal ganglia antibodies in 16 children with PANDAS and 40 neurological , streptococcal autoimmune, uncomplicated streptococcal infection and 30 healthy controls



There was a significant difference between mean absorbance in the PANDAS groups and controls using a Wilcoxon test. There was 47/220 (21%) of controls above the cut-off value for this ELISA (all data not shown as several data points overlapped). Therefore the specificity of the ELISA test was poor for PANDAS diagnosis although the sensitivity was adequate (83%). Statistics used were the Wilcoxon rank-sum test (Mann-Whitney), statistics summarised in table 4-6.

Table 4-6 Summary of PANDAS ELISA results

Group	Number	Mean absorbance	Confidence intervals	P value
PANDAS	16	0.410	0.341-0.471	-
Neurology controls	100	0.229	0.207-0.251	**p=<0.001**
Uncomplicated strep	40	0.189	0.171-0.207	**p=<0.001**
Strep autoimmune	50	0.251	0.200-0.302	**p=<0.01**
Normal controls	30	0.230	0.130-0.330	**p=<0.001**

p=value; Wilcoxon rank-sum test (Mann-Whitney), PANDAS versus controls.

4.15 Results of anti-basal ganglia ELISA in Tourette's syndrome

Introduction

Serum IgG was not tested as there was no ethical approval for this test which is a weakness of this study.

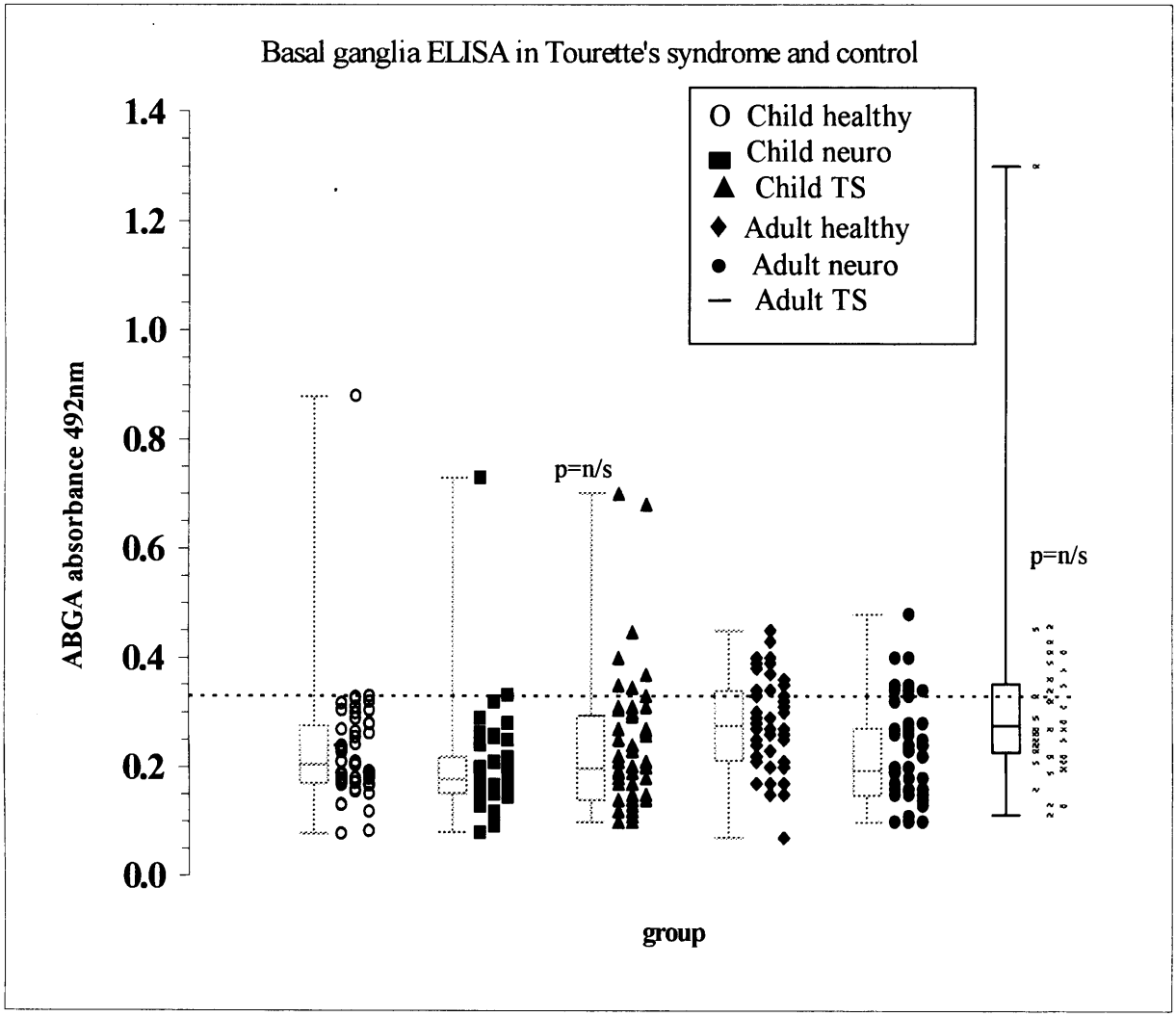
Results

The mean ABGA, ELISA result in the child TS groups was not different from the neurological and streptococcal infection controls (Figure 4-13). The mean ABGA, ELISA results in the adult TS group compared to the adult neurological disease and healthy controls was also not different (p=ns), (Figure 4-13).

Using the ELISA cut-off value (<0.33), an elevated ABGA ELISA result was only present in 16% (9/56) of the child TS cohort, 4% (2/50) of the neurology control group and 5% (2/40) of the streptococcal control group. There was no difference between groups (ns; p=0.09, p=0.08 respectively).

Using an ELISA cut-off of two standard deviations above the mean of both adult control groups (<0.43), an elevated ABGA ELISA was present in 11% (5/44) of adult TS, 2% (1/50) of adult neurological disease and 4% (2/50) of healthy controls, which gave no difference between groups (ns; p= 0.08, p=0.2), (Table 4-7). ELISA did not discriminate TS from control groups, (Table 4-7).

Figure 4-13 Box and whisker of 56 child and 44 adult Tourette's samples and 200 age matched controls



p=n/s; Wilcoxon rank-sum test (Mann-Whitney), PANDAS versus controls.

Table 4-7 Summary of Tourette's syndrome anti-basal ganglia antibodies ELISA results

Group	Number	Mean ABGA ELISA	SD	Confidence intervals	P value
Child TS	56	0.230	0.2	0.19-0.262	N/s
Child neurological disease controls	50	0.229	0.05	0.197-0.261	N/S
Child streptococcal infection controls	50	0.189	0.07	0.171-0.207	N/s
Adult TS	44	0.312	0.4	0.259-0.365	N/s
Adult neurological controls	50	0.224	0.06	0.197-0.251	N/s
Adult normal controls	50	0.282	0.02	0.259-0.305	N/s

P=n/s (not significant); Wilcoxon rank-sum test (Mann-Whitney), TS versus controls.

4.16 Absorbances in Sydenham's chorea, PANDAS and TS

Whilst a significant difference in ELISA was present in SC and PANDAS compared to controls the range of absorbances showed that ABGA is of low concentration (titre). This is because from the box and whisker plots, very few samples had absorbances over 1.0. Therefore like the IF results, ABGA ELISA show IgG responses are of low concentration in the blood.

4.17 Conclusions

The ABGA ELISA was designed to allow for comparison of IgG binding to basal ganglia antigens in large numbers of samples with SC, PANDAS and Tourette's and control groups. It was shown that highest ELISA results came from the ELISA prepared using a soluble S1 extract of basal ganglia homogenate. Background reactivity was lessened by removing lipids. Elevated IgG was not shown to be responsible for higher ELISA results in SC compared to controls. IgG was not measured in PANDAS or TS which is a major criticism.

Results are summarised in table 4-8. There was a significant difference in the mean ABGA ELISA in acute SC compared to control groups, although this was not the case with persistent SC where 40% were negative according to a numerical cut-off value. However, 95% of acute SC patients, using the same cut-off value had raised results compared to 5% of controls. Significant differences may imply a higher autoantibody or more likely a higher polyspecific antibody response in certain post-infectious groups which happen to have neurological sequeale.

There was a similar pattern of ELISA results in patients with PANDAS which are patients with acute onset, post-streptococcal tic disorder. However, antibody reactivity was higher in control patients with non-neurological, immune complications of streptococcal infection e.g. Nephritis, arthritis and rheumatic fever. Results raised above cut-off were present in 21% of all controls. This suggests that ABGA are common to post-streptococcal immune mediated disorders and are just a marker of disease, rather

than involved in pathology. Alternatively raised ELISA results may reflect binding to different epitopes in each group.

There was no significant difference in the mean ABGA ELISA results in either children or adults with TS compared to controls. This could mean that ABGA are not commonly present in TS or alternatively only a small subgroup is positive. Alternatively, as TS is a chronic disorder ABGA may only be a transitory phenonomen, present in the early stages of an inflammatory movement disorder perhaps only following recent infection. This probably suggests that anti-basal ganglia antibodies are just a marker of post-streptococcal neurology.

The ELISA might have been a useful screening test in acute onset post-streptococcal disorders SC and PANDAS but is of no use in Tourette's syndrome. The assay gave no indication of what the antigen was except for a soluble protein. A cytoplasmic antigen was suggested from IF studies which ELISA results support. Other autoantibodies may give false positive results. Contaminating factors such as heterophile antibodies may give false positive results. Whilst ELISA results in controls were below statistical cut-off from Brazilian controls this was not the case with UK controls groups, in the PANDAS study up to 21% of controls had raised results, although, this was mainly the streptococcal autoimmune patients without neurological signs.

In conclusion this assay is not recommended for the measurement of IgG auto-reactive antibodies against basal ganglia. Antibodies against a channel would also not be detected using this method so ELISA was not used further.

Table 4-8 Summary of ELISA results in post-streptococcal movement disorders

Group	Number	Mean absorbance	Number over cut-off (%) positive
Acute SC	34	0.62*	31/34 (91%) **
Persistent SC	24	0.40*	14/24 (58%) **
PANDAS	16	0.41*	12/16 (75%) **
Tourette's	56	0.23	9/56 (16%)
Normal control	40	0.21	0/40 (0%)
Neurological controls	50	0.23	2/50 (4%)
Rheumatic fever	20	0.21	2/20 (10%)
Streptococcal infection	40	0.19	2/40 (5%)
Streptococcal autoimmune disease	50	0.25	5/50 (10%)

*p=<0.01, Wilcoxon rank-sum test (Mann-Whitney), SC, PANDAS and TS versus controls
**p=<0.01, Fisher's exact test (2 tailed), Acute SC, PANDAS and TS versus controls.

5 Results of Western immunoblotting

5.1 Development of a method to detect anti-basal ganglia antibodies using Western immunoblotting

Introduction

Five separate striatum tissue blocks were used for this method. The first tissue used was optimised for detecting IgG responses in IF and ELISA positive samples. However, each tissue block was individually checked for optimum antigen dilution using 4 known positive and 4 negative samples detected using previous tissue. This was carried out using a 10 well PAGE gel containing double diluted antigen 1/2-1/128. Serum was diluted 1/300 and tested against Western blot. The antigen concentration which gave expected positive results without loss of reactivity seen with higher dilutions was used. The protein concentration on each blot was measured using a commercial kit (Biorad) and was between 30 µg and 50 µg of protein per gel, although less protein was on each blot.

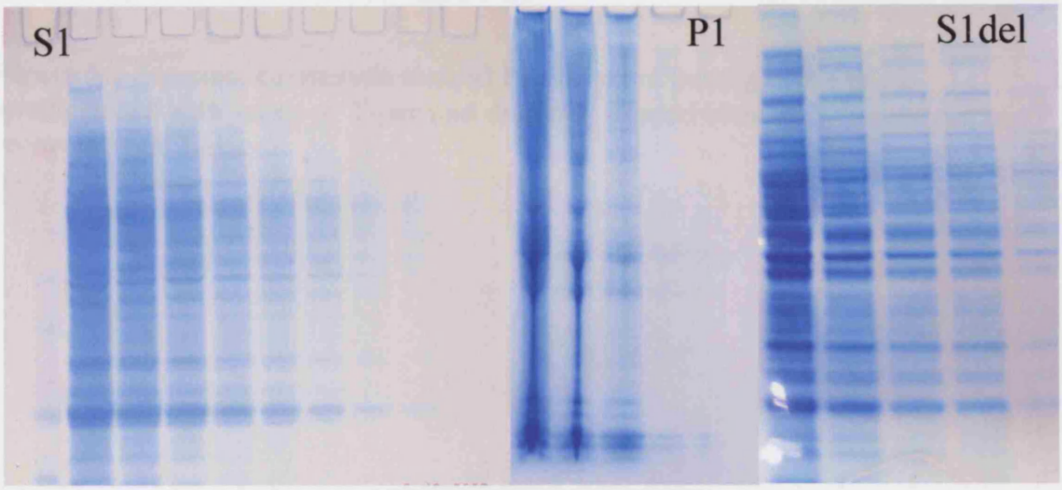
Two basal ganglia fractions were tested S1 and P1, although from ELISA and IF results the S1 fraction was likely to contain any reactive antigens. As T-per was used, membranous proteins would be present in this fraction as well. However, the remaining pellet P1 fraction was also studied. It is important to note that while the P1 fraction contained membranous proteins, denaturing the proteins for Western blotting could have damaged (denatured) any possible channel that might be a candidate antigen for ABGA.

5.2 Fractions tested for Western blotting

Prior to antigen concentration optimisation, fractions of human basal ganglia were tested by double dilutions of each fraction on a 10 well gel and stained. The first soluble fraction obtained from the crude homogenate S1 was collected as per ELISA antigen. This fraction was also treated by solvent extraction to remove lipid. The remaining pellet P1 was washed with Tris-HCL buffer and solublised using LDS sample buffer and heated at 65°C for 15 minutes. All fractions were double diluted (e.g. 1/2, 1/4, 1/8, 1/16) and PAGE was performed. The resulting Commassie stained gels are shown below (Figure 5-1).

Results: Page gel showing double dilution of the soluble S1 and remaining pellet P1of human basal ganglia. Multiple bands are present in the S1 fractions. The streaking in the S1 and P1 fractions is probably due to lipid and insoluble cell debris. Lipids were shown to be a problem in ELISA assay for ABGA. Therfore lipid was removed, gel was stained with high sensitivity Colloidal blue (commassie based) staining kit (Invitrogen) to show bands clearly (Figure 5-1 S1del).

Figure 5-1 Comassie stained gels S1 and P1 showing basal ganglia double diluted fractions and S1 delipidated gel stained with high sensitivity colloidal blue stain



S1 fraction, soluble cytosolic proteins, S2; P1 fraction remaining insoluble proteins and cell debris, all fractions were double diluted 1/2, 1/4, 1/8, 1/16 etc and 16µl loaded into each well. S1 del=soluble S1 fraction solvent extracted to remove lipid; stained with colloidal blue stain so bands can be seen in detail.

5.3 Influence of homogenisation methods on Western blots

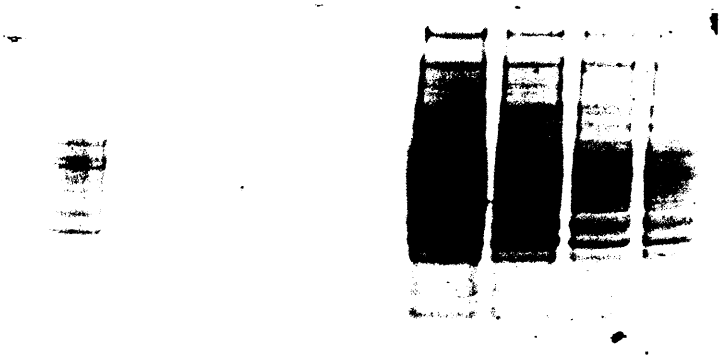
5.3.1 Protein recovery

The addition of T-per is thought aid the disruption of cell membranes, thereby increasing protein recovery (Perbio). However, many homogenisation methods include non-ionic detergents such as CHAPS, so T-per is just a commercial homogenisation reagent with the benefit of being reproducible. To assess whether T-per (Perbio) influenced results 2 tissue blocks were homogenised in saline or T-per. The supernatant was diluted (1/4-1/32), electrophoresed in a 10 well PAGE gel and stained with Commassie blue (Figure 5-2).

Results

There were more bands present in each doubling dilution with the T-per treated sample than those produced with saline (Figure 5-2).

Figure 5-2 Scanned, commassie stained PAGE gel of basal ganglia tissue homogenised with saline or T-per and doubling diluted showing increase protein recovery with T-per



MW Saline MW T-per

Soluble S1 fraction was obtained by homogenisation with saline or T-per, the results suggest T-per increases protein recovery

5.3.2 The influence of homogenisation on antibody reactivity

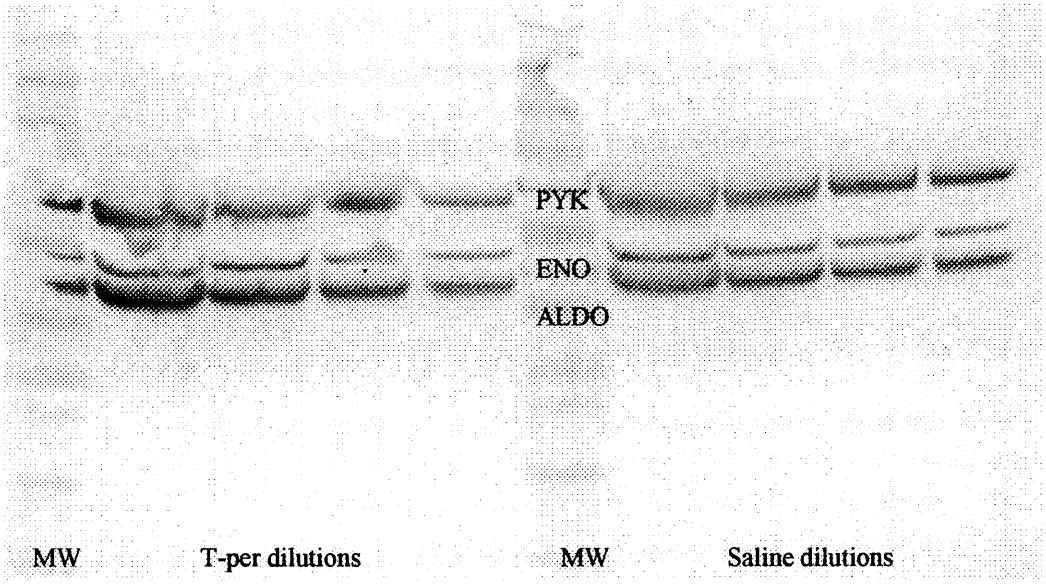
Introduction

To ensure that reactivity of protein was not affected by the addition of T-per, commercial antibodies against neuronal enzymes: Neuron-specific enolase, (ENO), (Autogen Bioclear), Pyruvate Kinase (PYK), (AbCam) and Aldolase C (ALDO), (AbCam) were diluted 1/5000 and tested against a 10 lane Western blot of soluble S1 fraction containing double dilution of antigens prepared with saline and T-per (Figure 5-3).

Results

There was no loss of antibody reactivity in the homogenate produced using T-per compared to saline (Figure 5-3).

Figure 5-3 Reactivity of commercial monoclonal antibodies against basal ganglia homogenates prepared using saline and T-per, showing no loss or change of reactivity with T-per



Commercial antibodies were tested against double diluted (1/2-1/16) S1 soluble fraction from basal ganglia, there was no difference in reactivity between saline and T-per fractions. T-per fractions appeared to give a stronger signal perhaps suggesting greater protein yield.

5.3.3 Antibody binding: colorimetric and chemiluminescence methods

Introduction

Visualisation of antibody binding was carried out using enhanced chemiluminescence (ECL), (Perbio, 34080) and colorimetric substrate: 4-chloro-1-naphthol (Sigma C8890), (Figure 5-4),

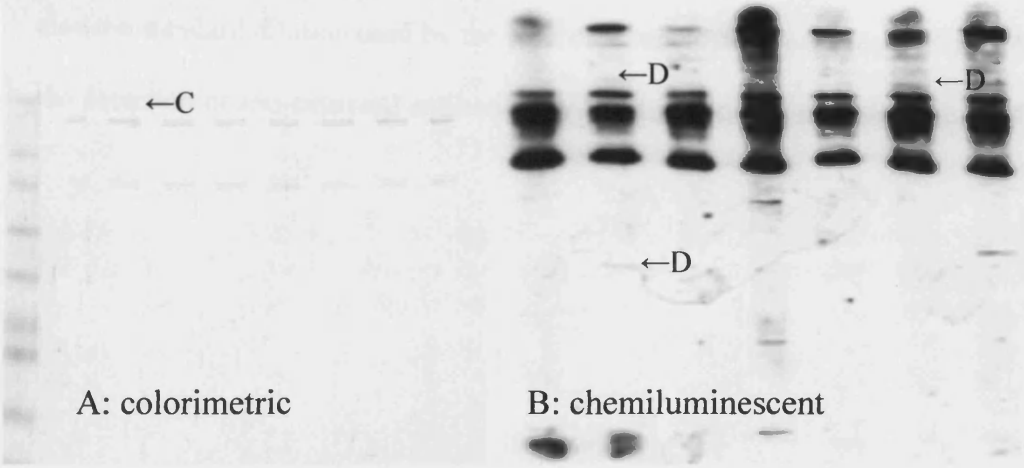
Results

ECL produced an array of bands in controls and patients with high (polyclonal) background, making analysis of specific responses difficult (Figure 5-4). The number of bands and extent of high background staining also depended on how long the

nitrocellulose was left developing with photographic paper, making timing very critical for the quality of results.

Colour development was more controllable and the stain less sensitive. This meant that weak affinity or low antibody concentration against brain tissue was less likely to result in multiple weak bands. Colour development of the Western blot was optimum at 15 mins. Longer development resulted in discolouration of the nitrocellulose making resolution of bands poor and scanning of the blots difficult. However, this method is less sensitive than ECL so weak reactivity may not be seen which a criticism of this method.

Figure 5-4 Sensitivity of basal ganglia antibodies developed using colorimetric or chemiluminescent techniques



1/300 diluted normal control samples tested; A: Secondary antibody 1/1000 for colorimetric; B: 1/5000 for ECL; C: Only one normal healthy control has a weak band on colorimetric development; D: Chemiluminescent development reveals multiple bands not present on colorimetric detection.

5.3.4 Stability of Western blotting homogenate

The prepared basal ganglia samples were stable (no loss of reactivity) for 48 hours at +4°C and 2 two weeks stored at -80°c. This was assessed by testing a positive SC sample known to be positive against a 60kDa band. The 60 kDa positivity appeared to be the same with the antigen source stored at +4°C for 2 days. The third day showed a decrease in reactivity and seven days later the band was barely visible. The activity of the 60 kDa band appeared to be unaffected by freeze-thawing as the LDS-treated sample kept for up to 2 weeks.

5.3.5 Dilution of secondary antibody

IgG binding was detected using rabbit anti-Human IgG (fab) conjugated with HRP (Dako). The secondary antibody was diluted in 0.9% saline, containing 0.1% fat free milk powder. The dilution of this antibody was always 1/1000; this is the recommended dilution for Western immunoblotting from the manufacturer (Dako). A 1/1000 dilution is also the standard dilution used by the Neuroimmunology Department, UCL NHS trust for the detection of anti-neuronal antibodies by Western blotting of brain tissue.

5.4 Antigen concentration of Western blotting of human basal ganglia

Introduction

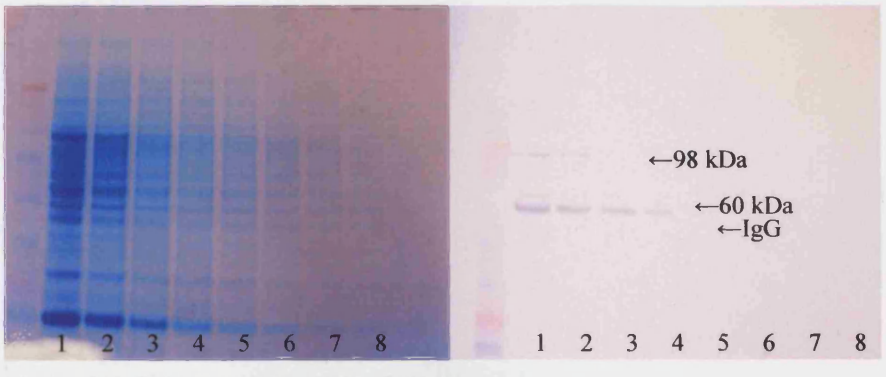
Four SC, ELISA and IF positive samples (2 weak and 2 strong) and 4 normal (no reactivity) controls were diluted 1/300 and tested against double dilutions of S1 and P1 fraction of human basal ganglia.

Results

None of the controls was positive, all four SC patients were positive against basal ganglia proteins. None of the SC patients of controls had any reactivity against the P1 fraction.

Thirty micrograms of protein 1/8 dilution (Figure 5-5) was the dilution of antigen used that gave positive results 60 and 98 kDa bands (Figure 5-5) without loss of bands present in higher concentrations in this experiment. As human tissue was used donor IgG was always present and tested by diluting secondary antibody 1/1000 and testing against the same blot as figure 5-5, 1 band at 50-55 kDa was commonly present (Figure 5-5).

Figure 5-5 Commassie stained gel and Western blot of titrated S1 fraction of human basal ganglia probed with positive Sydenham's chorea sample diluted 1/300 to assess protein concentration for Western blotting



Key: 1=1/4; 2=1/8; 3=1/16; 4=1/32; 5=1/64

The 1/8 dilution was used for all the following blots=30 µg protein per blot

Conclusions

Five tissue blocks from different cases were used for Western blotting previous Western blot positives from the last tissue block used were tested on a chequerboard of antigen dilutions (Figure 5-5) neat-1/128 to re-asses optimum antigen dilutions. This was always between 30 and 50 µg of protein per blot.

An important criticism is that it was assumed that any reactive antigens would be in first soluble S1 fraction because it contains detergent. However, a functional autoantibody would be likely directed against a channel. Whilst the membranous proteins are in both S1 and P1 fractions any channel reactivity is not usually apparent using Western blot. This method does not show that membranous proteins are not candidate antigens for antibodies in SC and PANDAS.

5.5 Optimum serum dilutions for Western blotting

Introduction

The results from both IF and ELISA showed that the expected titre of ABGA, or anti-neuronal antibodies is considerably lower than that of PNS anti-neuronal antibodies.

Results

Samples were not routinely titrated, instead a single point titration was used to analyse the presence of reactive proteins of the same molecular weights. In the Neuroimmunology laboratory sera are diluted 1/500 or 1/1000 for Western blotting and 1/500 for immunohistochemistry (Neuropathology department) for the detection of anti-neuronal antibodies for routine diagnosis of PNS. The optimum dilution of serum for the ABGA ELISA chequerboard was 1/300 and this same dilution was used throughout for Western blotting to compare the methods. Singer *et al* also used 1/250 dilution in both ELISA and Western blotting of human putamen and caudate (Singer *et al.*, 1998 and Singer *et al.*, 2003).

5.6 IgG reactivity using Western immunoblotting of S1 and P1 fractions of human basal ganglia in 14 patients with Sydenham's chorea and 14 normal controls

Introduction

Using colorimetric development of Western blot reactivity of 15 Sydenham's chorea and 14 normal controls to the S1 and P1 fraction of human basal ganglia was assessed.

Results

There was no reactivity in SC patients and controls against the P1 fraction of basal ganglia. (Number tested 14 in each group).

There was reactivity from SC samples to a number of bands in the S1 fraction (Figure 5-7), which was expected from the ELISA and IF results, as antigens appeared to be cytoplasmic and therefore likely soluble. Bands were absent in controls (Figure 5-6).

Conclusion

The S1 fraction was used to test basal ganglia antibodies as the P1 fraction gave no binding. The soluble nature of the antigens means a membranous antigen is unlikely. This makes a functional effect of anti-basal ganglia antibodies unlikely as functional antibodies are considered to be only reactive against membrane proteins such as in LEMS or Myaesthenia Gravis. ABGA might still be a marker of disease though.

5.7 Western blot results in Sydenham's chorea and controls using 30 µg of soluble S1 fraction of basal ganglia and 1/300 dilution of serum

Introduction

Using the optimum Western immunoblotting method of S1 fraction and 1/300 dilution of serum 58 SC, 20 RHF and 40 normal controls were tested.

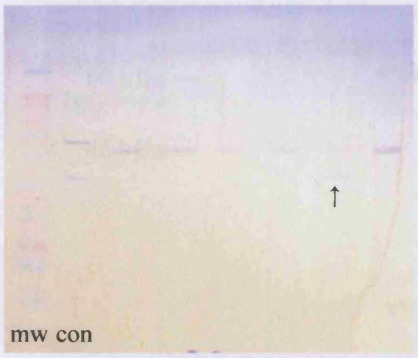
Results

97% (33/34) of acute SC, 63% (15/24) of persistent SC, 83% (48/58) of the entire SC cohort and 10% (2/20) of RHF controls reacted against at least 1 basal ganglia protein (Table 5-1). None of the healthy controls 0% (0/40) had any binding (Figure 5-6 and 5-7). There was reactivity to a number of basal ganglia antigens with molecular weights of: 35 kDa (n=2), 40 kDa (n=15), 45 kDa (n=13), 60 kDa (n=7), 80 kDa (n=2) and 98 kDa (n=3).

The most common antigens were 40, 45 and 60 kDa. In the acute SC group only 15% (3/20) did not react with any of the 3 common basal ganglia antigens (Figure 5-8). The most common antigens in the persistent SC group were also 40 kDa (n=6), 45 kDa (n=5) and 60 kDa (n=6). All of the positive persistent SC reacted to at least 1 of these antigens (Figure 5-8).

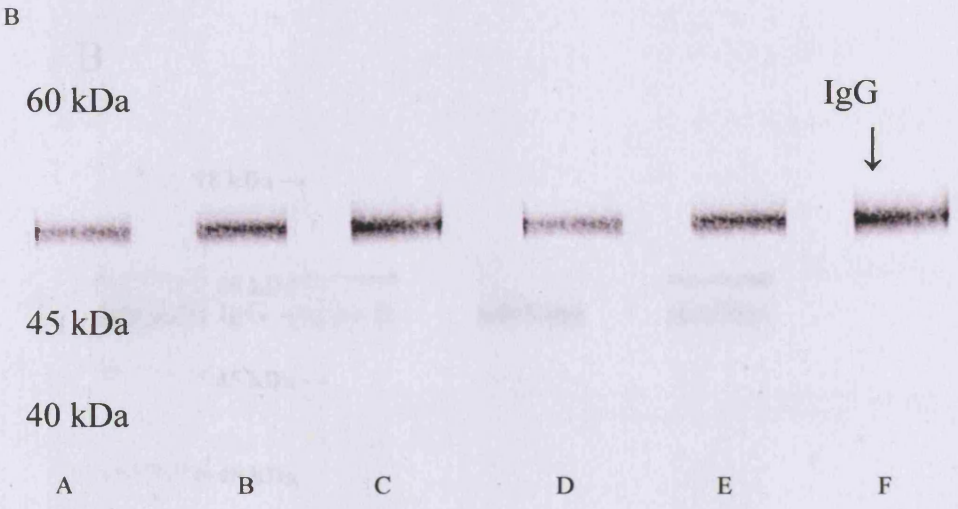
Two patients with RHF who were ELISA and IF positive reacted to a 40 kDa antigen (Figure 5-6). Native IgG from the human tissue donor was detected in the basal ganglia homogenate (Figures 5-6, 5-7 and 5-8), (Table 5-1).

Figure 5-6 Western immunoblotting of 6 control samples tested against basal ganglia protein



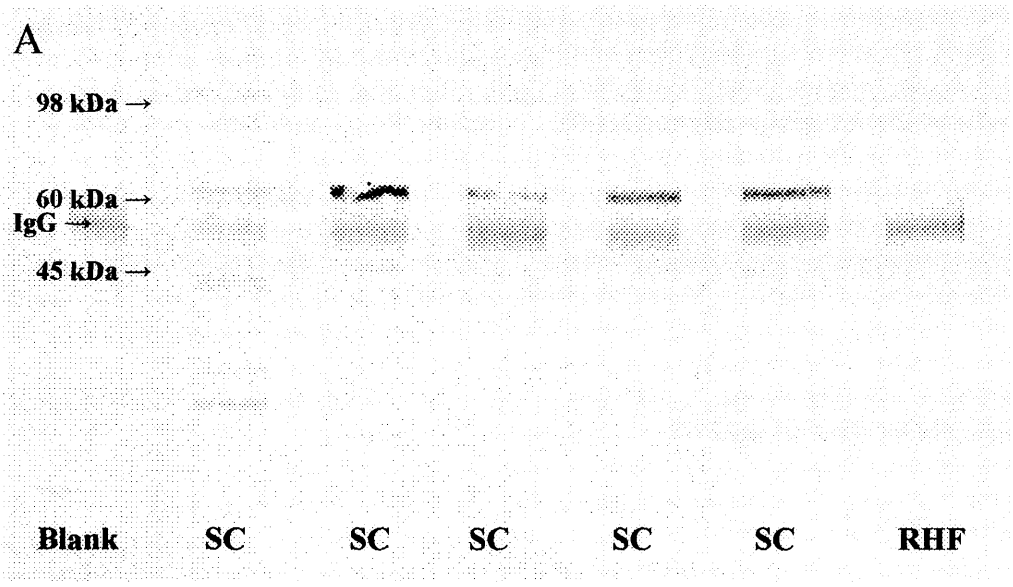
Mw=molecular weight marker, con=positive control
lanes 1, 2 and 3 normal controls,
lanes 4, 5 and 6 RHF, lane 5 40 kDa band 9arrow)

Figure 5-7 Detailed Western blot showing no binding of 3 normal controls and 2 rheumatic fever controls to 40, 45 or 60 kDa bands

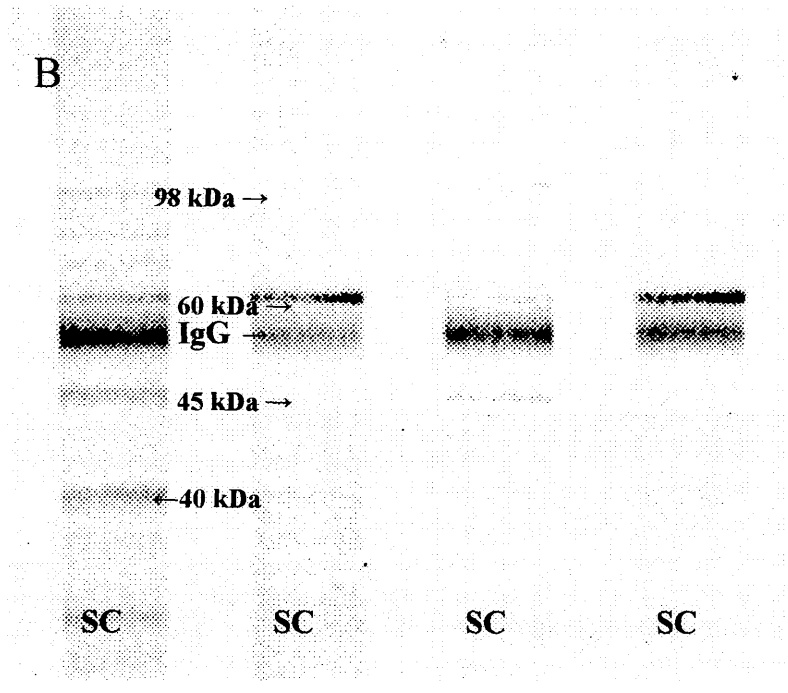


B: Detailed figure of normal controls tested against a soluble S1 fraction og human basal ganglia showing no reactivity except IgG from donor. A: 2nd antibody Rabbit anti-Human IgG HRP conjugated only; B, C and D: Normal control with no specific bands; E, F: Rheumatic fever control with no specific bands

Figure 5-8 Western immunoblotting of 6 Sydenham's chorea patient tested against basal ganglia proteins



A: Results show strong staining against a 60 kDa protein in all patients with weaker responses to 45 kDa antigen in 3 patients. The first patient had multiple bands against two antigens at approximately 40 kDa, and 1 weak band against 60 kDa and one at 98 kDa. The reactivity of the IgG detector antibody against donor IgG is present in all wells, as expected. Rheumatic fever control was negative.



B: Four SC patients with multiple bands of reactivity, but with common 40, 45, 60 and 98 kDa

The reactivity of the IgG detector antibody against donor IgG is present in all wells, as expected.

Table 5-1 Summary of Western immunoblotting results in the Sydenham's chorea cohort

Group	Positive/ number	P value	Basal ganglia antigens (molecular weights)
Acute SC	33/34	-	30 kDa (n=1), 35 kDa (n=2), 40 kDa (n=15), 45 kDa (n=13), 50 kDa (n=1), 60 kDa (n=7), 80 kDa (n=2), 95 kDa (n=1), 98 kDa (n=3)
Persistent SC	15/24	0.0009	40 kDa (n=6), 45 kDa (n=5), 50 kDa (n=2), 60 kDa (n=6), 80 kDa (n=3), 90 kDa (n=1), 95 kDa (n=2), 100 kDa (n=1)
Rheumatic Fever	2/20	<0.0001	40 kDa (n=2), 95 kDa (n=1)
Normal controls	0/30	<0.0001	No binding

P value; Fisher's exact test (2 tailed), Acute SC versus controls and persistent SC. Persistent SC versus RHF p= 0.0005 and normal controls p=<0.001.

5.8 Sydenham's chorea cerebrospinal fluid anti-basal ganglia antibodies by Western immunoblotting

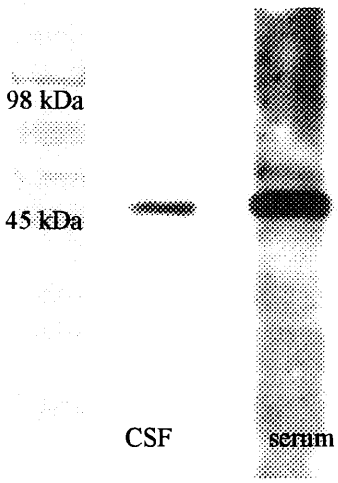
Introduction

Neat CSF of 6 acute and 5 persistent SC patients was of sufficient volume to be tested for ABGA.

Results

In acute SC, 83% (5/6) and persistent SC 100% (5/5) had IgG reactivity against basal ganglia antigens. The basal ganglia antigens recognised by ABGA were the same in CSF as serum for each patient tested. The antigens common to CSF and serum IgG in acute SC were 35 kDa (n=1), 40 kDa (n=5), 45 kDa (n=3), 60 kDa (n=5), and 80 kDa (n=1). The persistent SC CSF and serum responses were 45 kDa (n=3), 60 kDa (n=4), 80 kDa (n=1), and 95 kDa (n=1), (figure 5-9).

Figure 5-9 Serum diluted 1/300 and neat cerebrospinal fluid from 1 patient with SC tested against 30 µg of S1 fraction of human basal ganglia



SC CSF and serum binds to the same 45 kDa protein

5.9 Sydenham's chorea patients from the UK, Western immunoblotting results of anti-basal ganglia antibodies

In the acute SC group recruited from the UK, 92% (11/12) of patients had positive reactivity to at least 1 of the common Brazilian SC antigens (40, 45, 60, 98 kDa), (Table 24). Reactivity to the same antigens was present in both the UK and Brazilian, SC cohorts. In addition, reactivity against 38, 80 and 95 kDa basal ganglia proteins was seen in 4 acute SC patients. These additional responses were unusual and inconsistent. One acute SC patient had no reactivity against any Western immunoblotting protein and was also IF negative. In the persistent SC patients, 33% (1/3) reacted to antigens at 40kDa (n=1), 60 kDa (n=2) and 98 kDa (n=1), (Table 5-2). The persistent SC samples did not bind to any other basal ganglia proteins, although the numbers tested were small since SC is a rare condition in the UK.

Western immunoblotting of basal ganglia produced a specific 60 kDa band in 1 control with post-streptococcal arthritis. This patient had no evidence of RHF or movement disorder, although no psychiatric assessment was performed. Two controls with post-streptococcal glomerulonephritis reacted against antigens of 20 and 55 kDa in 1 patient, and 60 kDa in the other. There was no evidence of a movement disorder although the psychiatric history was not assessed in detail. None of the patients with post-streptococcal vasculitides had reactivity to basal ganglia but 1 patient from the RHF group bound to a 40 kDa antigen, although again the number of patients tested was small (Table 5-2).

Table 5-2 UK Sydenham's chorea and controls Western blot results

Samples	Number (sex M:F)	Western blot Positive/negative (%)	P value
Developmental delay controls	30 (15/15)	0/30, (0%)	P=<0.0001
Post-streptococcal arthritis	15 (8/7)	1/15, (6%)	P=<0.0001
Post-streptococcal glomerulonephritis	14 (5/9)	2/14, (14%)	P=0.0002
Post-streptococcal vasculitides	15 (5/10)	0/15, (0%)	P=<0.0001
Rheumatic fever	5 (1/4)	1/5, (20%)	P=0.0099
Acute Sydenham's chorea	12 (2/10)	11/12, (93%)	-
Persistent Sydenham's chorea	3 (1/2)	2/3, (66%)	-

Fisher's exact test (2 tailed), Acute SC versus controls. No statistics were performed on the persistent SC group as the numbers tested were too small for meaningful comparison

5.10 Titration of four strongly positive Sydenham's chorea samples against 30 micrograms of S1 fraction of human basal ganglia

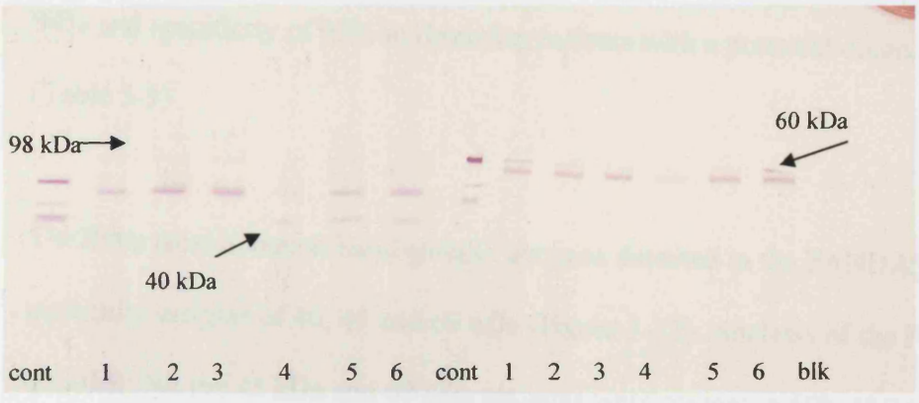
Introduction

Forty positive SC samples diluted 1/300 were visually assessed for reactivity on Western blot.

Results

Only 4 samples from 40 (10%) were considered to have strong binding and these samples were serially diluted 1/300, 1/500, 1/1000 and tested against a Western blot of human basal ganglia (Figure 5-10). Three samples had binding at 1/500, and 1 at 1/1000.

Figure 5-10 Western blots showing serial dilution of strong positive post-streptococcal movement disorder patients



con: control, 1: 1/300, 2: 1/500, 3: 1/1000
blk: blank, 6: 1/300, 5: 1/500, 4: 1/1000

Conclusions

In accordance with IF and ELISA only a minority of patients have reactivity at titres greater than 1/500.

5.11 Western immunoblotting results in 16 PANDAS patients and 220 controls

Introduction

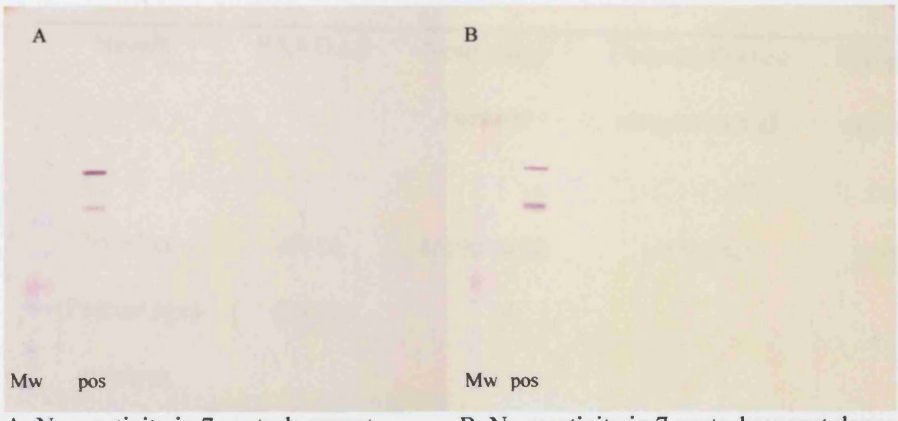
Western immunoblotting demonstrated antibody reactivity to specific basal ganglia antigens in the PANDAS patients rather than a polyspecific response

Results

Reactivity against any basal ganglia antigen was significantly more prevalent in the PANDAS patients compared to any of the controls ($p<0.0005$), (Fisher's exact test), (Figures 5-11 and 5-12). Analysis of the patients and controls found that reactivity to basal ganglia protein was present in 94% (15/16) of the PANDAS patients compared to 5% (11/220) of all the control groups combined. This method derived a sensitivity of 94% and specificity of 95% in detecting patients with a potential diagnosis of PANDAS (Table 5-3).

The three most common basal ganglia antigens detected in the PANDAS patients had molecular weights of 40, 45 and 60 kDa (Figure 5-12). Analysis of the PANDAS results revealed that the 45 kDa and 60 kDa antigens were present in 50% (8/16). In contrast, reactivity to the 40 kDa antigen was present in 62.5% (10/16) of the PANDAS group (Table 5-3).

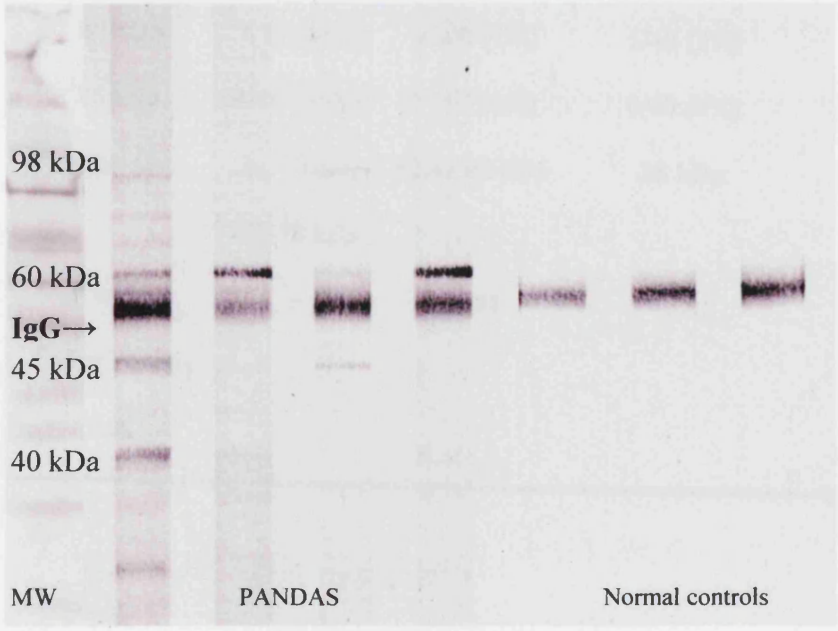
Figure 5-11 Fourteen neurological disease controls tested 1/300 against Western blots of S1 fraction of human basal ganglia



A: No reactivity in 7 controls expect Donor IgG reactivity as expected
Mw: molecular weight marker
Pos: positive control 40, 45 and 60 kDa

B: No reactivity in 7 controls except donor IgG reactivity as expected
Mw: molecular weight marker
Pos: positive control to 40, 45 and 60 kDa

Figure 5-12 Antibody binding of four PANDAS sera and three normal controls on a Western immunoblot of S1 fraction of human basal ganglia



Western blot showing 4/4 PANDAS patients with antibody to a 60 kDa protein, 1 with 40 kDa binding, 2 with 45 kDa, 2 with 98 kDa. Common bands were present due to reactivity of the secondary antibody with IgG from the donor tissue. Normal controls showed no reactivity.

Table 5-3 Summary of PANDAS and control Western blotting results

Result	PANDAS	Neurology control	Uncomplicated streptococcal	Streptococcal autoimmune controls	Normal controls
Number (Percentage) positive	15/16 (94%)	4/100 (4%)	1/40 (2%)	5/50 (10%)	1/30 (3%)
Mw:					
40 kDa	8/16 (50%)	1/100 (1%)	0/40 (0%)	2/50 (4%)	0/30 (0%)
45 kDa	7/16 (44%)	0/100 (0%)	0/40 (0%)	0/50 (0%)	0/30 (0%)
50 kDa	0/16 (0%)	0/100 (0%)	0/40 (0%)	0/50 (0%)	1/30 (3%)
60 kDa	7/16 (44%)	1/100 (1%)	1/40 (2%)	1/50 (2%)	0/30 (0%)
80 kDa	4/16 (25%)	0/100 (0%)	0/40 (0%)	0/50 (0%)	0/30 (0%)
other mw	30, 35, 42, 62, 98 kDa	42,43,95 kDa	35 kDa	35, 50 kDa	50 kDa
P value, Fisher's exact test	-	<0.01	<0.01	<0.01	<0.01

5.11.1 Results of Western blots in control groups

Four percent (4/100) of the neurological control group were ABGA positive with Western immunoblotting. The clinical presentation of the ABGA positive patients were: Status dystonicus (n=1), encephalitis (n=1), ataxia (n=1) and leukodystrophy (n=1). Only the sample from the patient with encephalitis bound to the common 40 and 60 kDa antigens seen in SC and PANDAS. The other patient samples, bound to a number of different antigens (42, 43, 95 kDa). Two percent (1/40) of the uncomplicated streptococcal infection controls were ABGA positive and bound to 35 and 60 kDa antigens. Three percent (1/30) of the normal controls was also positive and bound to a 50 kDa antigen. Ten percent (5/50) of the autoimmune were also positive (Table 5-3). Two patients had RHF: one reacted to a 50 kDa basal ganglia antigen whilst the other reacted to a 40 and 60 kDa antigen. Two patients with glomerulonephritis reacted to 35 and 40 kDa antigens and 1 patient had arthritis and also reacted to a 40 kDa antigen. All the ABGA positive autoimmune controls had post-streptococcal autoimmune diseases (Table 5-3).

5.12 Western blotting results in 100 patients with Tourette's syndrome and 100 controls

5.13 Results from paediatric Tourette's syndrome

Common bands of reactivity were seen in the TS group rather than polyspecific binding.

Results

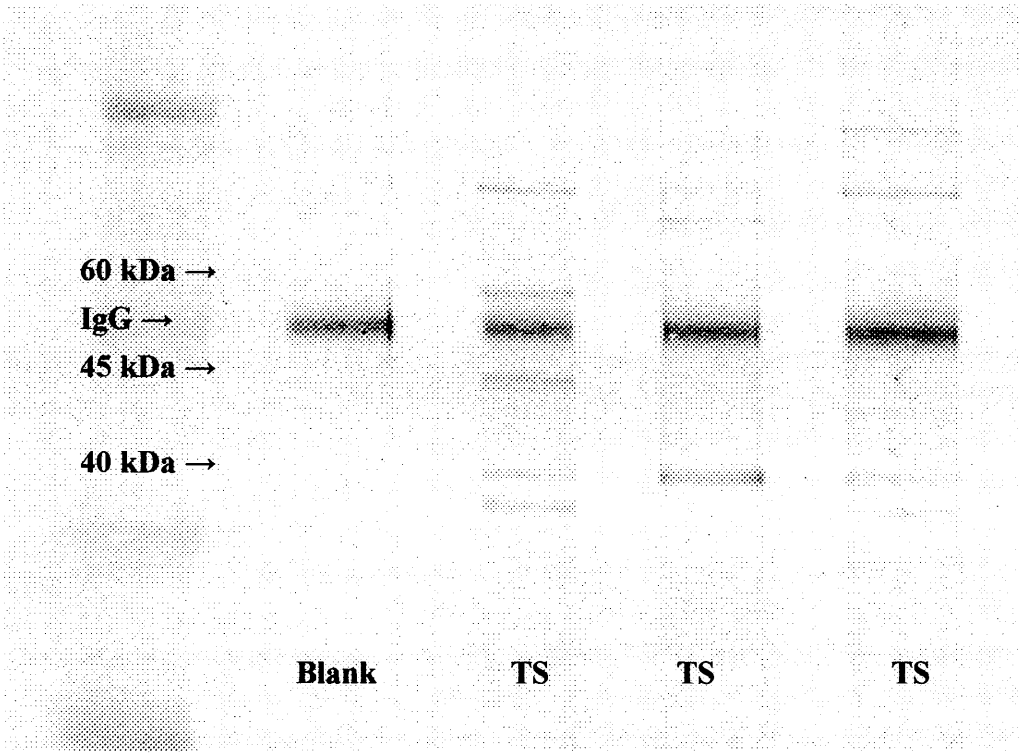
Twenty percent (12/56) of the paediatric TS group had positive Western immunoblotting, compared to 4% (1/50) of the neurological controls and 2% (1/40) of the streptococcal controls. This was significantly different in the TS group compared to both control groups ($p<0.05$ and $p<0.05$ respectively), (Fisher's exact test). The most common response was to 60 kDa (n=6), 40 kDa (n=4), 45 kDa (n=3), 67 kDa (n=2), 80 kDa (n=2) and 95 kDa (n=1) antigens (Figure 5-13). The positive neurological controls bound to a 40 kDa (n=1) and 43 kDa (n=1) antigen. The positive streptococcal control bound to a 60 kDa antigen.

5.13.1 Results from adult Tourette's syndrome

Twenty seven percent (12/44) of the adult TS group had positive Western immunoblotting which was higher when compared to 2% (1/50) of neurological controls and 4% (2/50) of healthy controls, ($p<0.005$ and $p<0.005$ respectively), (Fisher's exact test). The common antibody binding was also to 60 kDa (n=7), 40 kDa (n=5), 45 kDa (n=2), 80 kDa (n=2), 67 kDa (n=1) and 98 kDa (n=1) antigens. The positive neurology control bound to a 40 kDa antigen. The positive healthy controls both bound to a 55 kDa

antigen. In all paediatric and adult TS patients the most common basal ganglia autoantigens detected were to a 60 kDa protein (n=13) followed by 40 kDa (n=9).

Figure 5-13 Three positive samples from Tourette's syndrome patients tested against the S1 fraction of human basal ganglia



5.14 Homogenised guinea pig brain as a source of antigen for Western blotting to identify anti-neuronal antibodies in post-streptococcal movement disorder patients

Introduction

Guinea pig homogenate is used by the Neuroimmunology department, University College Hospital NHS Trust, London to perform Western blotting for the identification of paraneoplastic anti-neuronal antibodies. Therfore to confirm the 40, 45 and 60 kDa reactivity in SC and PANDAS, Western blots of 50 μg S1 fraction of homogenised guinea pig brain was tested using 7 normal, 14 neurological disease controls, and 3 strongly positive Sydenham' and 2 PANDAS samples. All sera were diluted 1/300.

Results

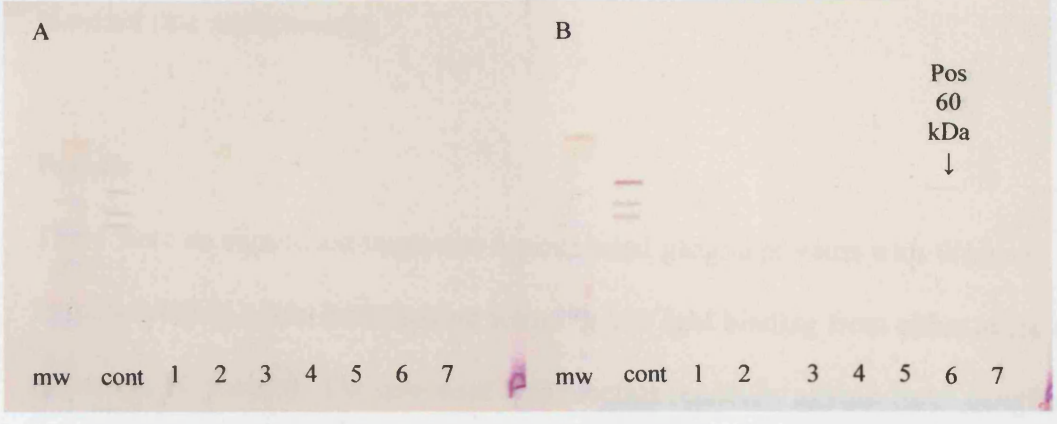
Results from controls, SC and PANDAS are shown in Figure 5-14. One control with cerebellar ataxia was positive to a 60 kDa band. All SC samples were positive to a 60 kDa band which was found on human basal ganglia; however 1 patient only had very weak binding which was difficult to show on an image. One PANDAS patient was positive and 1 was negative.

Conclusion

These results only show a point of principal that animal brain might be used to detect IgG reactivity against the bands common to SC and PANDAS. A large number of samples need to be tested. Not all of the strong positive IgG binding against human basal ganglia proteins was reflected in the results from guinea pig brain. This might reflect antigen

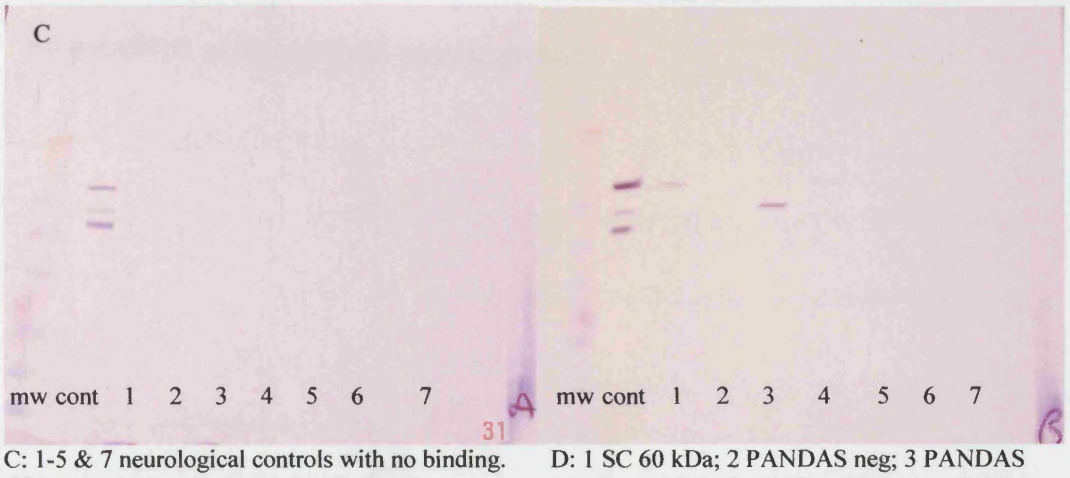
concentration, localisation and source. However, reactivity to 45 and 60 kDa proteins were present so whole animal brain could be used as an antigen source but requires methological assessment.

Figure 5-14 Western blot of a soluble fraction of guinea pig brain probed with 7 neurological disease control samples and 7 psychiatric disease control was diluted 1/300



A: mw= molecular weight marker
Cont: 40, 45 and 60 kDa positive
7 normal controls with no binding

B: mw= molecular weight marker
cont=40, 45 and 60 kDa positive
6 neurological controls negative, 1 positive to 60 kDa with cerebellar ataxia



C: 1-5 & 7 neurological controls with no binding.
6 Neurological control with 80 kDa band,

D: 1 SC 60 kDa; 2 PANDAS neg; 3 PANDAS 45 kDa; 4 SC 35 & 60 kDa; 5 SC trace 60 kDa; 7. Blank

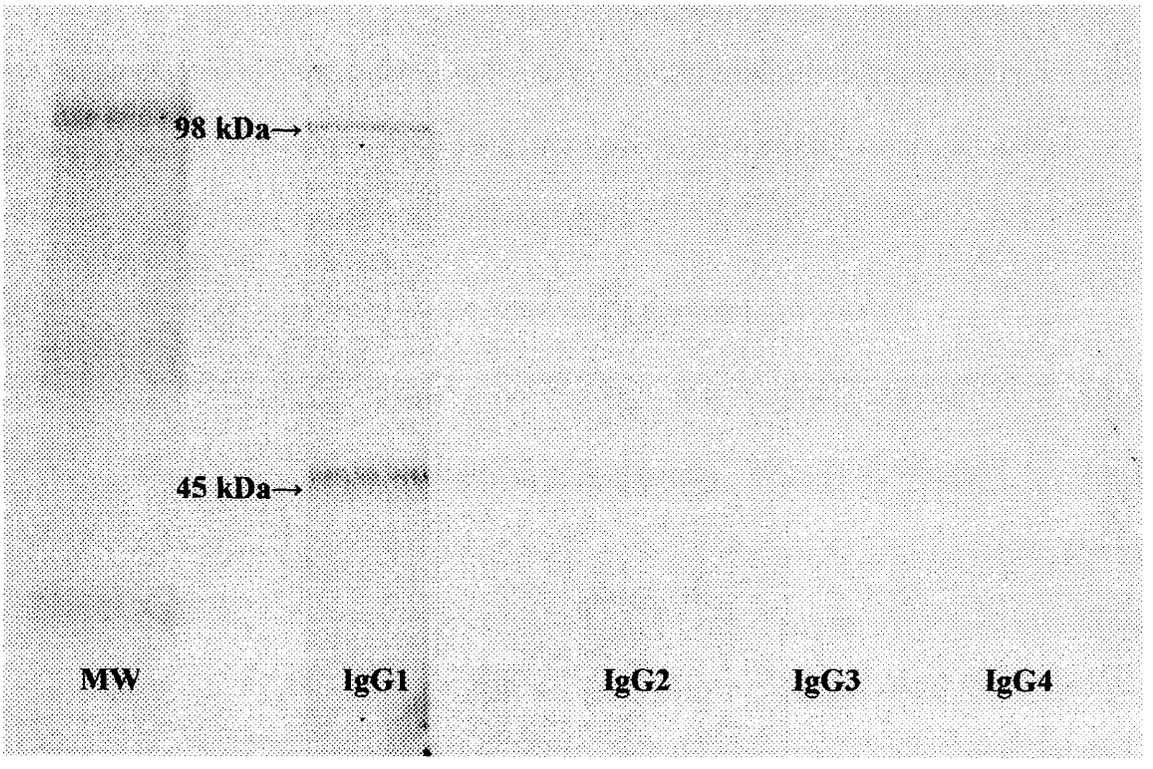
5.15 Identification of the dominant immunoglobulin type and IgG subclass specificity of ABGA binding using Western immunoblotting in 7 SC patients

Serum from 5 acute SC patients and 2, persistent SC patients had the same pattern of binding to basal ganglia antigens on Western immunoblotting (40, 45 and 60 kDa), when staining for different IgG subclass binding. None of these samples had abnormal immunoglobulin levels above the ranges appropriate for their ages, measured using standard rate nephelometry.

Results

There were no significant responses against basal ganglia proteins with Western immunoblotting when investigating serum IgA or IgM binding from either acute or persistent SC patients. The dominant IgG subclass reactivity against basal ganglia proteins was IgG_1 or IgG_3 in both acute and persistent SC (Figure 5-15). None of the patients tested had any reactivity when using IgG_2 or IgG_4 antiserum against Western immunoblots of human basal ganglia proteins.

Figure 5-15 IgG subclass responses from a patient with Sydenham's chorea against basal ganglia proteins



5.16 Adult movement disorders: UK study

The results from this study showed that patients with known genetic movement disorders (dystonia) were negative for ABGA and had no evidence of raised streptococcal serology. In patients with primary blepharospasm, which has been reported to be associated with autoimmunity, were included in this study (Jankovic *et al* 1993). Evidence of ABGA was also not found, but 1 patient had raised ASOT (10% 1/7), (Table 5-4). This is in accordance with the expected incidence of raised ASOT in an adult healthy population as seen in these adult TS studies.

In comparison, 25% of patients with idiopathic dystonia were ABGA positive by IF and Western immunoblotting, but only 4 had a raised ASOT. Unfortunately anti-DNAse B was not measured in these patients as the assay was not available at the time. Interestingly, 60% of patients with adult onset tics or "Tourettism" were also ABGA positive. ASOTs were raised in all of these patients.

Table 5-4 Results in patients serum from adult movement disorders

Diagnosis	**Number**	**ABGA IF % positive**	**ABGA WB % positive**	**ABGA ELISA Mean (% > 0.33)**	**ASOT Mean (% >200 IU/ml)**
Primary blepharospasm	7	0% (0/10)	0% (0/10)	0.213 (0%)	152 IU/mL (10%, 1/10)
DYT1 positive dystonia	10	0% (0/10)	0% (0/10)	0.199 (0%)	135 IU/mL (0%, 0/10)
Idiopathic dystonia	20	25% (5/20)	25% (5/25)	0.38 (30%, 6/30)	230 IU/mL (20%, 4/20)
Huntington's disease	3	0% (0/3)	0% (0/3)	0.299 (33%, 1/3)	96 IU/mL (0%, 0/3)
Tourettism	5	60% (3/5)	40% (2/5)	0.30 (20%, 1/5)	200 IU/mL (40%, 2/5)

5.17 The sensitivity and specificity of the anti-basal ganglia antibody assays in Sydenham's chorea

Results of Western blotting are summarised in table 5-5.

Results

The sensitivity and specificity of the ABGA IF method was calculated in all the SC samples tested (Brazilian and UK) and found to be 96% (44/46) in acute and 63% (17/27) in persistent SC. The specificity of IF in SC was 96% (5 positive controls from 139 tested). The sensitivity of the ABGA ELISA was 89% (41/46) in acute and 55% (15/27) in persistent SC. The specificity of the ELISA was 95% (6 positive controls in 139 tested). The sensitivity of the ABGA Western Immunoblotting was 96% (44/46) in acute and 59% (16/27) in persistent SC. The specificity of Western immunoblotting was 95% (6 positive controls from 139 controls). The majority of controls were ABGA negative (non-diseased or patients with development delay (70/139).

Table 5-5 Summary of Western blotting results

Group	Number	Common bands	Number positive (%)	Most common band
Acute SC	46	40, 45, 60, 98 kDa	44/46 (96 %) **	45 kDa
Persistent SC	27	40, 45, 60, 98 kDa	16/27 (59%) **	40, 60 kDa
PANDAS	16	35, 40, 45, 60, 80 kDa	15/16 (94%) **	40 kDa
TS	56	40, 45, 60, 67, 80, 95 kDa	12/56 (20%) **	60 kDa
Normal control	40	-	0/40 (0%)	-
Neurological controls	100	40, 42, 43, 60, 95 kDa	4/100 (4%)	-
Rheumatic fever	20	40, 95 kDa	2/20 (10%)	40 kDa
Streptococcal infection	40	35, 60 kDa	1/40 (2%)	35 kDa
Streptococcal autoimmune disease	44	20, 55, 60	5/50 (10%)	55 kDa

**p=<0.01, Fisher's exact test (2-tailed). Acute, persistent SC, PANDAS and TS versus controls

5.18 Diagnostic utility of anti-basal ganglia antibodies in idiopathic movement disorders

As Western immunoblotting was highly sensitive and specific in post-streptococcal movement disorders, the method was evaluated as a diagnostic tool in children with new onset, otherwise 'idiopathic' tic disorders. Those patients with a clinical diagnosis of post-streptococcal tics (n=16) and chorea (n=20) were compared with a dystonic, neurological disease control group (n=32). The diagnostic sensitivity of ABGA was 93% and specificity 97%. The positive predictive value was 97% (likelihood ratio for a positive test = 31), and the negative predictive value was 91% (likelihood ratio for a negative test = 0.072). There was common binding to basal ganglia antigens of 40, 45, and 60 kDa. There was no difference in the antigen binding pattern between tic or chorea phenotypes using the 45 kDa and 60 kDa antigens. In contrast, reactivity to the 40 kDa antigen did appear to be more predominant in the patients with tics rather than chorea as reactivity to this antigen was present in 62.5% (10/16) compared to 30% (6/20) of the chorea patients ($p < 0.001$).

5.19 Conclusions

Western immunoblotting was a reliable method for detecting IgG responses against soluble basal ganglia protein. There was a significant difference between SC, PANDAS, TS and the various normal and diseased controls tested. Colorimetric development was better than ECL. In accordance with IF and ELISA antibody titres were low compared to paraneoplastic antibodies. A standard dilution of 1/300 appeared to be discriminatory of patients with post-streptococcal neurology and any control groups studied, including patients with non-neurological, post-streptococcal sequalae. The discriminatory power of Western blot appeared to be superior to ELISA as the positivity in controls was lower with Western blot.

Western immunoblotting confirmed the presence of IgG responses in SC, PANDAS and a subgroup of patients with TS. The basal ganglia antigens with the most reactivity were from the soluble S1 fraction, so ABGA might be a marker of disease rather than functional as the antigens are not membranous but soluble. A 1/300 dilution was used to screen multiple samples and controls so antibodies are low titre. Titration of many positive samples to assess the end-point titre of antibody would be useful. Any future work could investigate the end point titres of ABGA in each group but this wasn't done and that identification and characterisation of the S1 antigens was the main aim of this thesis.

6 Other Laboratory evidence

Introduction

To assess the role of the immune system in Sydenham's chorea and if pursuing ABGA as a potential disease marker of this disease was worthwhile other surrogate markers of immunity were investigated in Sydenham's chorea including cytokines and oligoclonal bands.

6.1 Results of cytokine studies in serum and cerebrospinal fluid from patients with Sydenham's chorea

Results

There was only a modest elevation of any cytokine in the CSF and serum of acute SC patients. As expected, there was no elevation in cytokine levels compared to normal in the neurological disease control group.

Serum results

Serum IL-4 was above the normal limit in 23% of acute SC whilst IL-10 was elevated in 46% and IL-12 69% of acute SC (Table 6-1). In comparison, no elevation in cytokines was shown in the serum samples from the persistent SC group (Table 6-2). The mean serum levels of IL-4, IL-10 and IL-12 were all elevated in the acute SC group compared to the persistent SC group and controls ($p=<0.001$), (Wilcoxon rank-sum test). Serum INF-γ was not detectable in any of the SC groups or controls (Tables 6-1 and 6-2).

Table 6-1 Serum cytokine levels in Acute Sydenham's chorea; mean concentrations and ranges

Group	Acute SC mean pg/mL (range)	Normal range pg/mL	Proportion above normal range
Serum IFN-γ	0.7 (0-1.1)	<1.5	0%
Serum IL-4	3.9 (2.3-6.8) *	<4.6	23%
Serum IL-10	20 (0-63) *	<10	46%
Serum IL-12	94 (0-377) *	<30	69%

*(p=<0.01); Wilcoxon rank-sum test (Mann-Whitney).

Table 6-2 Serum cytokine levels in persistent Sydenham's chorea; mean concentrations and range

Group	Persistent SC mean pg/mL(range)	Normal range pg/mL	Proportion above normal range
Serum IFN- γ	Not detectable (<0.6)	<1.5	0%
Serum IL-4	2.50 (0.2-3.4)	<4.6	0%
Serum IL-10	Not detectable (<0.5)	<10	0%
Serum IL-12	5.2 (0-22)	<30	0%

P=n/s; Wilcoxon rank-sum test (Mann-Whitney).

CSF results

The normal upper-limit of cytokines in CSF was calculated from the mean level of cytokine in the neurological disease control group. In the CSF analysis, IL-4 was above the normal limit in 31% of acute SC (Table 6-3). Fifty percent of persistent SC cases also had raised IL-4, whilst CSF IL-10 was also elevated in 31% of acute SC but 0% of persistent SC (Table 6-4). Compared to neurological controls there was an increase in CSF IL-4, but not other cytokines in both acute and persistent SC ($p<0.01$). CSF IL-10 was only raised in acute SC compared to controls ($p<0.01$). The concentrations of IFN-γ were also not significantly elevated in either CSF or serum or any SC group or controls (Tables 6-3 and 6-4).

Table 6-3 CSF cytokine levels in acute Sydenham's chorea; mean concentrations and ranges

Group	Acute SC mean pg/mL (range)	Control mean pg/mL (range)	Proportion above control mean
CSF IFN-γ	Not detectable (<0.6)	Not detectable (<0.6)	0%
CSF IL-4	3.0 (2.6-3.7) *	1.5 (0.5-2.5)	31%
CSF IL-10	1.9 (0-8) *	Not detectable (<0.5)	31%
CSF IL-12	N/A	N/A	N/A

*p=<0.01; Wilcoxon rank-sum test (Mann-Whitney).

Table 6-4 CSF cytokine levels in persistent Sydenham's' chorea; mean concentrations and ranges

Group	Persistent SC mean pg/mL (range)	Control mean pg/mL (range)	Proportion above control mean
CSF IFN- γ	Not detectable (<0.6)	Not detectable (<0.6)	0%
CSF IL-4	3.0 (2.8-3.3) *	1.5 (0.5-2.5)	50%
CSF IL-10	Not detectable (<0.5)	Not detectable (<0.5)	0%
CSF IL-12	N/A	N/A	N/A

* p=<0.01; Wilcoxon rank-sum test (Mann-Whitney).

6.2 IgG oligoclonal bands in Sydenham's chorea

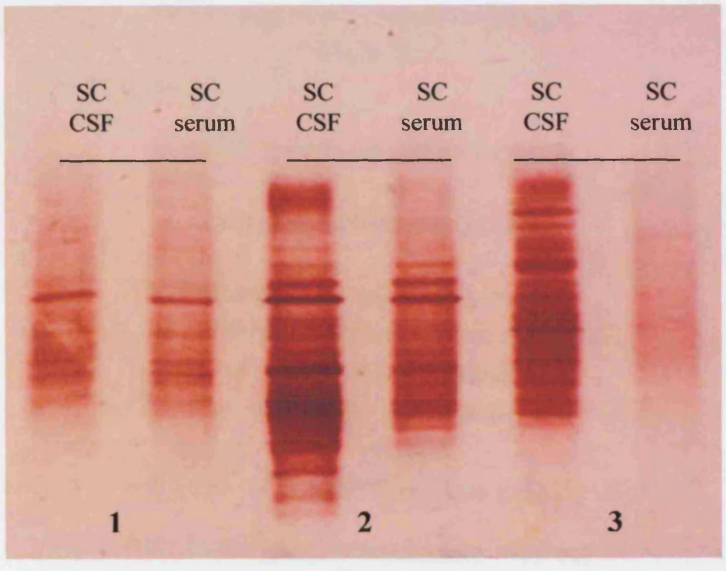
Introduction

Oligoclonal bands are a useful laboratory test for diagnosis inflammatory neurological diseases such as MS and encephalitis.

Results

In the acute SC group 6 of 13 (46%) had the presence of oligoclonal IgG bands. Two patients had intrathecal synthesis only (CSF OCB) whilst 4 patients had a mirrored pattern of OCB (Serum and CSF matched OCB). The patients with detectable OCB had the highest levels of cytokines but did not reach statistical significance compared to OCB negative results (p=ns). There was no difference in the pattern of cytokines from the other OCB negative patients. All persistent SC patients and neurological controls were OCB negative. Typical results are presented in Figure 6-1.

Figure 6-1 Three paired CSF and serum from patients with acute Sydenham's chorea Typical range of oligoclonal band results present in Sydenham's chorea



1=Mirror pattern of sample bands in serum and CSF; 2= Mirror pattern but extra bands in CSF; 3=Bands in CSF only, intrathecal synthesis.

6.3 Clinical comparisons between PANDAS and Sydenham's chorea in the UK

There was no difference in mean age of onset in either PANDAS or SC studied during 1999-2003 (Table 6-7). There was however, a male preponderance in PANDAS (tics and neuropsychiatric symptoms), (Table 6-7). In comparison patients with SC were more likely to be female (Table 6-7). Patients with PANDAS were more likely to have continuation of the movement disorder on follow up ($p=<0.01$). There was no difference in individual psychiatric manifestations between PANDAS and SC, although any psychiatric symptom was more common in patients with PANDAS.

Table 6-5 Patients from the UK with PANDAS and Sydenham's chorea: clinical and laboratory findings

Characteristic	PANDAS (n=16)	Chorea (n=20)
Mean age of presentation (range)	6.75 (2-13)	7.85 (1.5-13)
Male %	69%	35%
Length follow-up- mean (range)	3.84 (0.2-13)	1.1 (0.2-7)
Continuing movement disorder	94%*	55%
Psychiatric co-morbidity-total	69%*	45%
• Obsessive-compulsive disorder	50%	10%
• Generalised anxiety	19%	25%
• Major depression	12.5%	20%
• ADHD	25%	15%
• Conduct disorders (all)	25%	20%
Anti-basal ganglia antibodies indirect Immunofluorescence	94%	90%
Anti-basal ganglia antibodies Western immunoblotting: antibody binding		
• Positive binding	94%	95%
• 40 kDa	62.5%*	30%
• 45 kDa	50%	40%
• 60 kDa	44%	45%
• 80 kDa	12.5%	25%

P=<0.001; Fisher's exact test (2 tailed). PANDAS versus Sydenham's chorea.

Conclusions

There was no difference in the antigen binding pattern between PANDAS or chorea using the 45 kDa and 60 kDa antigens. In contrast, reactivity to the 40 kDa antigen did appear to be more predominant in the patients with PANDAS rather than chorea as reactivity to this antigen was present in 62.5% (10/16) compared to 30% (6/20) of the chorea patients ($p < 0.001$). Whilst there is some evidence for immune activation in SC this was not persuasive for an autoimmune syndrome

7 Streptococcal serology results

Introduction

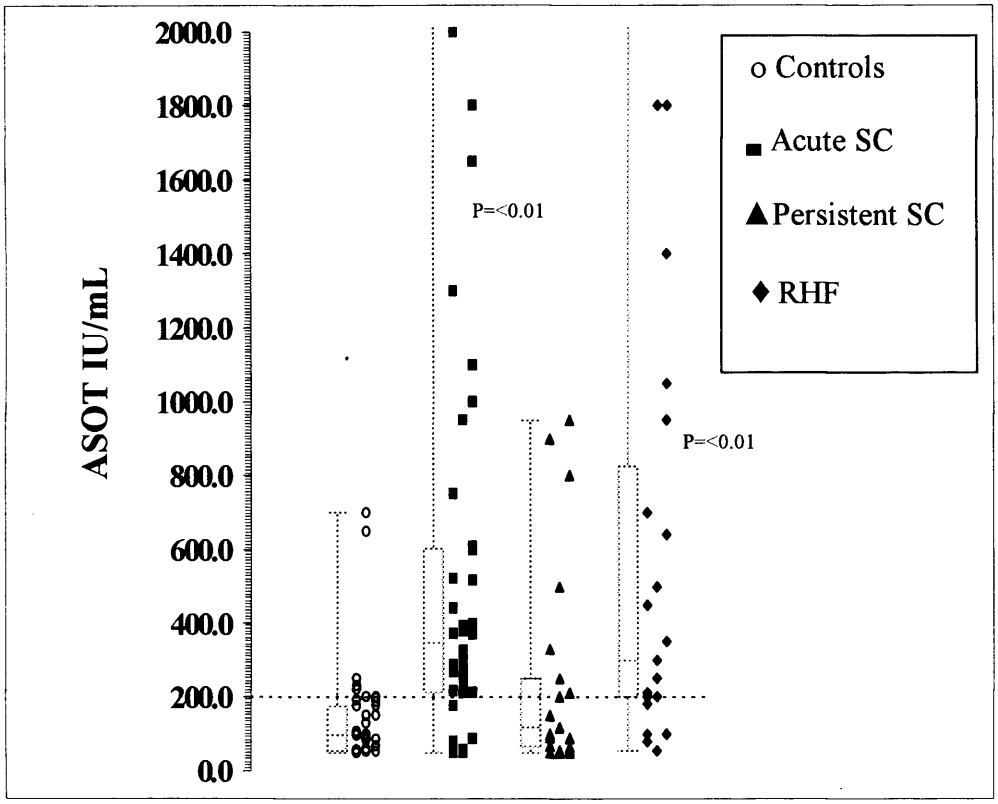
Streptococcal serology was investigated to assess the presence of GABHS in each group of patients.

7.1 Streptococcal serology in all Sydenham's chorea samples and controls (Brazilian cohorts)

The results of the ASOT serology were plotted in a box-and-whisker graph for Brazilian SC cohorts and controls collected during 1999-2002. The line represents the median ASOT and the whisker is the range of results (Figure 7-1). The median values were used for statistical comparisons as the data was not normally distributed. The data was not normalised as ASOT is a WHO standardised assay and variability of antibody response to streptolysin O was expected.

7.2 Anti-streptolysin O titre

Figure 7-1 Anti-Streptolysin O titres in 40 acute, 24 persistent Sydenham's chorea and 40 normal and 25 Rheumatic fever controls



P=<0.01 acute SC and RHF vs. controls and RHF; Wilcoxon rank-sum test (Mann-Whitney).

Results of median ASOT comparisons

The median ASOT was 349 IU/mL in the acute SC group and 188 IU/mL in the persistent SC group. In the normal control group the median ASOT was 97 IU/mL whilst the RHF group was 300 IU/mL. In both the acute SC and RHF groups there was an increased ASOT compared to the normal control group (P=<0.01 in both instances). The ASOT level in the acute SC group was not different compared to the RHF group (p=n/s).

The mean ASOT in the persistent SC group was not increased compared to normal controls or acute SC or RHF.

Results of comparisons of patients with controls using normal cut-off levels

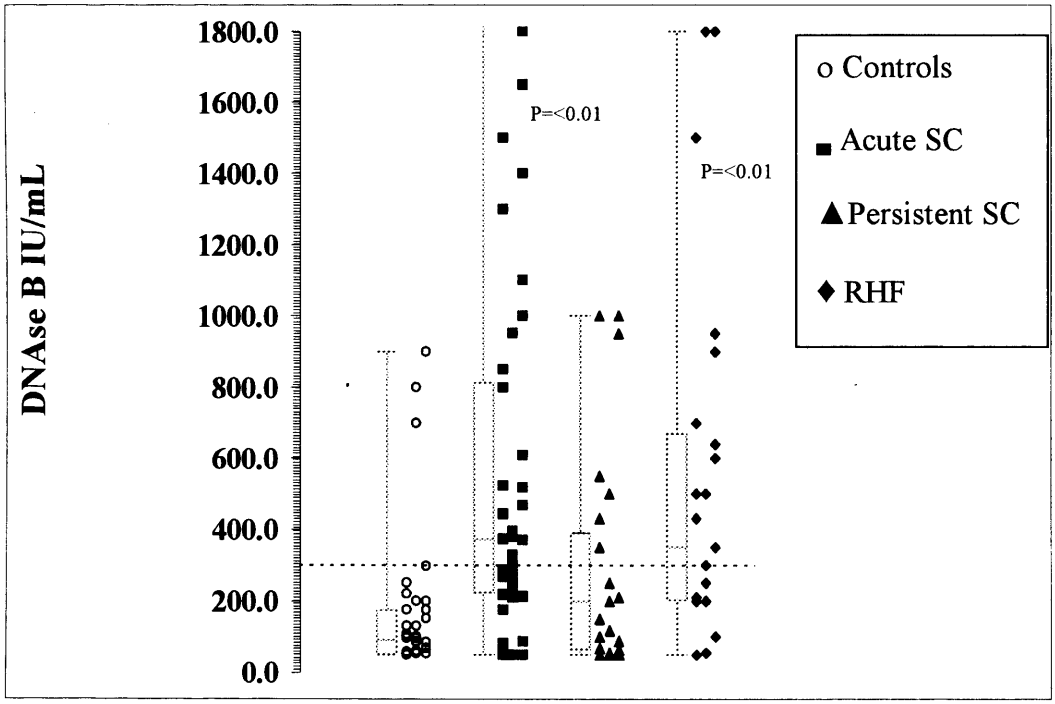
Using the normal WHO cut-off value for normal ASOT levels (<200IU/mL); 72% (18/25) of the RHF group, 75% (30/40) of acute SC, 29% (7/24) of persistent SC and 15% (6/40) of the control group had raised ASOT levels (>200 IU/mL). The numbers of patients with raised ASOT in the acute SC and RHF group was increased compared to both persistent SC and controls (p=<0.001, p=<0.001, Fisher's exact test). There was no difference in the percentage of ASOT positive results in persistent SC compared to controls (p=0.2). There was also no difference in the number of ASOT positive samples between RHF and acute SC. Unfortunately throat cultures were not routinely taken in the Brazilian SC cohorts.

7.3 Anti-DNAsè B titres

Introduction

The results of the anti-DNAse B serology were plotted in a box-and-whisker graph for both Brazilian SC cohorts and controls collected during 1999-2002. The line represents the median DNAse B results and the whisker is the range of results (Figure 7-2). The median values were used for statistical analysis as the data was not normally distributed.

Figure 7-2 Anti-DNAse B titres in 40 acute, 24 persistent Sydenham's chorea and 40 normal and 25 Rheumatic fever controls the Sydenham's chorea cohort (Brazilian)



P=<0.01; Wilcoxon rank-sum test (Mann-Whitney).

Results of median anti-DNAse B comparisons

The median anti-DNAse B result in the acute SC group was 372 IU/mL and persistent SC was 200 IU/mL. In the control groups the RHF group had a median anti-DNAse B titre of 350 IU/mL whilst in the normal controls it was 92 IU/mL. The acute SC and RHF groups both had increased median anti-DNAse B levels compared to the normal control groups (P=<0.01 in both instances).The median anti-DNAse B in acute SC was not different from the mean anti-DNAse B in the RHF group. The median anti-DNAse B level in the persistent SC group was not different from normal controls (p=0.06) or acute SC or RHF (p=n/s).

Results of comparisons of patients with controls using normal cut-off levels

Using 300 IU/mL as a normal upper-limit cut-off value, 60% (15/25) of the RHF group, 65% (25/40) of the acute SC, 36% (9/24) of persistent SC and 10% (4/40) of the control group had raised anti-DNAse B levels. The numbers of patients with raised DNAse B levels in the acute SC and RHF groups was increased compared to both persistent SC and controls (p=<0.01, p=<0.01, Fisher's exact test). There was an increase proportion of patients with raised DNAse B positive results in the persistent SC group compared to normal controls (p=0.03). There was also no difference in the number of DNAse B positive samples between RHF and acute SC groups.

7.3.1 Comparison of elevated ASOT and DNAse B titres in Sydenham's chorea

None of the acute SC patients had both negative ASOT and anti-DNAse B. There was no preponderance of either raised ASOT or DNAse B in acute SC. The persistent SC cases that were ABGA positive (15/24) had either raised ASOT or DNAse B whilst two

patients who were ABGA negative (2/9) had raised ASOT in one case and raised anti-DNAse B in the other. Neither ASOT nor anti-DNAse B was more likely to be raised in the persistent SC patients who were ABGA positive. The ASOT was raised in 66% (7/15) and anti-DNAse B in 60% (9/15) of the persistent ABGA positive patients (p=ns). One patient with persistent SC was positive for both ASOT and anti-DNAse B. There was no difference in the amount of time chorea had been present in the persistent SC cases, so this did not influence the ASOT results.

7.4 Evidence for Streptococcus in Sydenham's chorea and controls from the UK

7.5 Results of throat swab for the culture of Streptococcus from 17 UK patients with Sydenham's chorea

In the UK group 3/13 (23%) of the acute SC patients tested were GABHS positive on throat culture. None of the 4 persistent SC patients were GABHS positive. One patient thought to have persistent SC, was later found to have chorea of a vascular pathogenesis and was ASOT and anti-DNAse B negative.

Further evidence for streptococcal infection was obtained from clinical history (RHF and pharyngitis). Streptococcal serology was used as surrogate marker of recent infection. There was no ethical approval for measuring streptococcal serology in UK patients with development delay.

Results of median serology comparisons

The childhood controls with mixed neurological diseases where ASOT had been measured for the TS study, had a median ASOT of 151 IU/mL with 18% (9/50) having raised levels (>200IU/mL). This was lower than both UK SC and other streptococcal autoimmune controls (p=<0.01 in all instances). Streptococcal serology was measured in the SC and streptococcal autoimmune controls to see whether there was any differences in serology between phenotypes (CNS versus systemic).

7.6 Anti-streptolysin O titres

Introduction

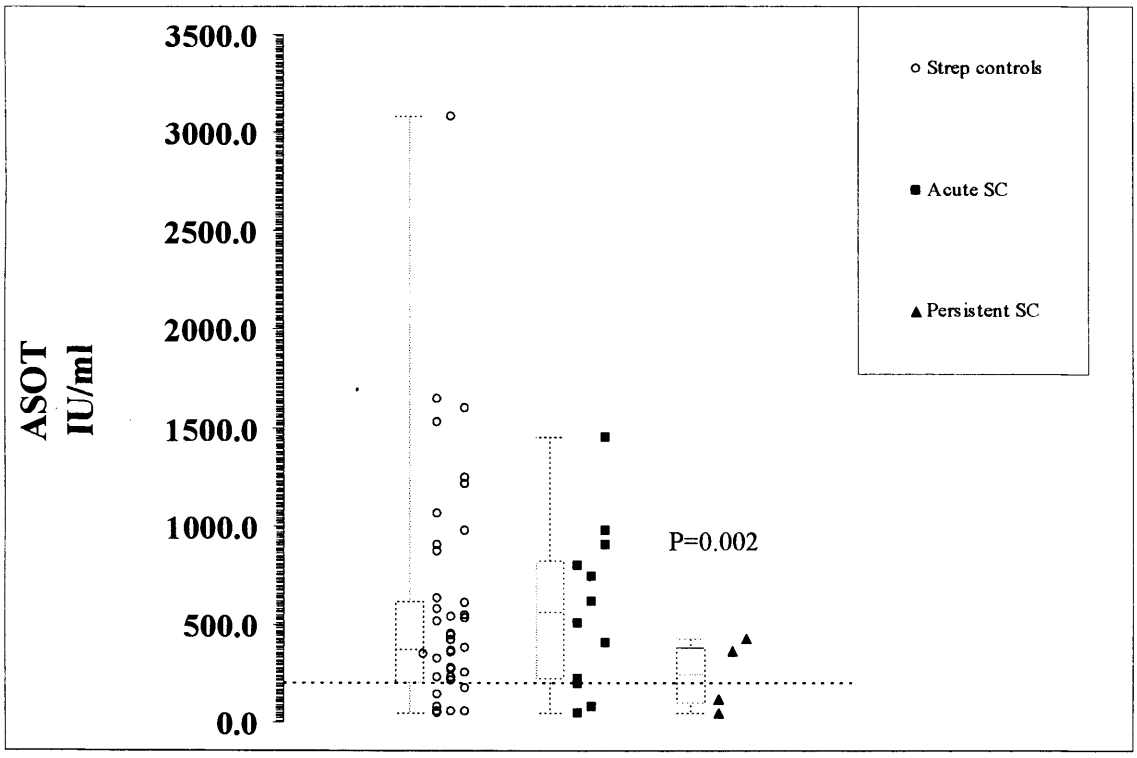
Box-and-whisker plot of ASOT in both UK SC cohorts and controls collected during 1999-2002, (Figure 7-3). The line represents the median ASOT result and the whisker is the range of results. The median values were used for statistical analysis as the data was not normally distributed.

Results

The combined streptococcal autoimmune disease controls had a median ASOT of 480 IU/mL (SD 584) compared to the acute SC group median (618 IU/mL) and was not significantly different (p=ns), (Figure 7-3). The persistent SC group, although small in number, had a median ASOT level of 330 IU/mL which was lower than both streptococcal controls and acute SC (P=<0.002), (Figure 7-3). This finding was similar to the Brazilian persistent SC cohort. In the streptococcal autoimmune controls 75% (33/44)

had ASOTs greater than 200 IU/mL whilst in the acute SC patients 85% (11/13) had raised levels (p=ns). In the persistent SC group the numbers were too small for a meaningful comparison but there appeared to be a decrease in the number of patients with raised ASOT, i.e. 50% (2/4). The small numbers tested is a criticism of this study.

Figure 7-3 Anti-Streptolysin O titres in 13 acute, 4 persistent Sydenham's chorea and 44 Streptococcal controls from the UK



p=0.002; Wilcoxon rank-sum test (Mann-Whitney).

7.7 Sydenham's chorea patients in the UK: Anti-DNAse B results

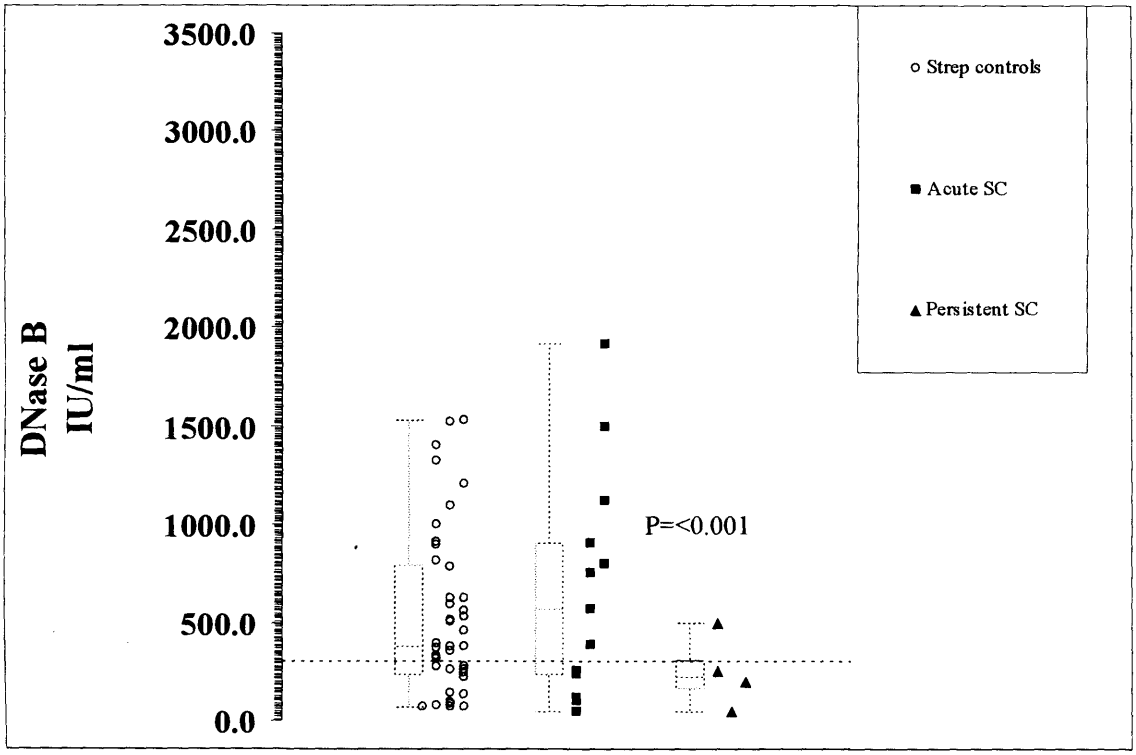
Introduction

Box-and-whisker plot showing the results of anti-DNAse B serology for both UK SC cohorts and controls collected during 1999-2002. The box represents the median anti-DNAse B result and the 10-90% percentile of results; whilst the whiskers show the 1st and 99th percentiles of the anti-DNAse B results (Figure 7-4).

Results

The anti-DNAse B results showed a similar pattern to the ASOT levels. The median anti-DNAse B in acute SC was 670 IU/mL and persistent SC 190 IU/mL. In the streptococcal controls it was 422 IU/mL and there were no differences between anti-DNAse B in controls and acute SC (p=ns). However, anti-DNAse B in the persistent SC group was lower, although the numbers tested were too small to make statistically significant comparisons. Using the upper-limit of normal for anti-DNAse B, 66% (29/44) of the streptococcal, autoimmune controls were positive, comparable to the acute SC group (62%, 8/13). The anti-DNAse B in the persistent SC group was again lower; only 25% (1/4) of patients had raised levels (Figure 7-4).

Figure 7-4 Anti--DNAse B results in 13 acute, 4 persistent Sydenham's chorea and 44 Streptococcal controls from the UK



p=<0.001; Wilcoxon rank-sum test (Mann-Whitney).

7.8 Recrudescent Sydenham's chorea

The median ASOT was 190 IU/mL (50-210 IU/mL). One patient (1/5, 20%) had a raised ASOT of 210 IU/mL. The median anti-DNAse B level was 255 IU/mL (50-290IU/mL). No patient had raised anti-DNAse B. One patient (1/5, 20%) was subsequently diagnosed with Coeliac disease.

7.9 Streptococcus and PANDAS

7.10 Results of throat swab for the culture of Streptococcus from 16 UK patients with PANDAS

GABHS was cultured from the throat in 31% (5/16) of the PANDAS patients. All patients (16/16) had a preceding illness compatible with a streptococcal pharyngitis. No patient had clinical or laboratory signs of RHF. The latency between infection and onset of tics and psychiatric symptoms was 2 weeks (2-47 days).

7.11 Streptolysin O and DNAse B in PANDAS and controls

Introduction

A box and whisker plot was not done as the numbers of PANDAS patients tested was small. However, mean ASOT was used to assess statistical differences due to the large numbers of control groups investigated. The streptococcal serology was positive (ASOT, anti-DNAse B or both) in all PANDAS patients (Table 7-1).

Table 7-1 Summary of streptococcal serology in PANDAS and controls

Result	PANDAS	Neurology controls	Uncomplicated streptococcal infection	Streptococcal autoimmune controls	Normal controls
Number	16	100	40	50	30
Mean ASOT IU/mL	505	151	348	621	130
ASOT 95% confidence intervals	(415-599)	(102-200)	(269-427)	(351-891)	(50-180)
Mean DNAse B IU/mL	580	213	423	576	220
DNAse B 95% confidence intervals	(410-436)	(128-298)	(346-500)	(474-678)	(75-305)
P value, (Wilcoxon non-parametric test)	-	<0.001	n/s	n/s	<0.001

Results

The mean ASOT in the PANDAS patients was elevated compared to the neurological and normal controls (p=<0.001), (Table 7-1). There was no difference in the mean ASOT compared to uncomplicated streptococcal infections and autoimmune complications of streptococcal infection (p=ns), (Table 7-1). The pattern of results was the same when testing anti-DNAse B. There was an increased mean DNAse B level in PANDAS compared to neurological and normal controls (p=<0.001) but not streptococcal controls (p=ns).

7.12 Streptococcal serology in Tourette's syndrome

7.13 ASOT results from paediatric Tourette's syndrome

Only ASOT was measured due to unavailability of the anti-DNAse B assay at the time of the study. Mean ASOT was elevated in the child TS group compared to the neurological controls ($p<0.001$), but not the child streptococcal infection controls ($p=0.4$), (Figure 7-5, Table 7-2). Mean ASOT was also elevated in the adult TS cohort compared to the neurological disease ($p=0.04$) and healthy adult controls ($p=0.04$), (Figure 7-5, Table 7-2).

7.13.1 Analysis of the number and percentage of Child Tourette's and controls with raised ASOT serology

The number and percentage of cases with raised ASOT (<200 IU/mL) in the child streptococcal controls (80%) and child TS (64%) were different compared to children with neurological disease (18%), ($p=<0.01$, $p=<0.01$, Fisher's exact test). The number of children with raised ASOT after recent streptococcal infection (80%) was higher than the number with raised ASOT in TS (64%), ($p=0.04$), (Table 7-2).

7.13.2 ASOT results from adult Tourette's syndrome

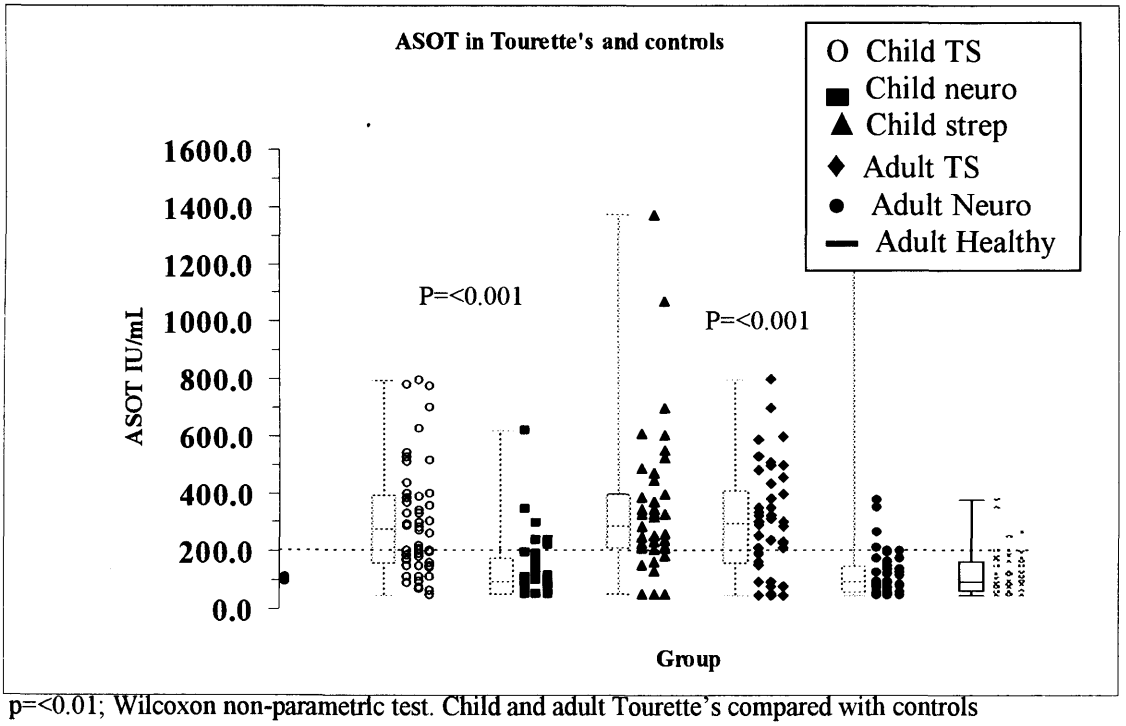
The number of cases with raised ASOT in the adult TS group (68%) was higher than in the neurological disease (12%) and healthy adult (8%) groups ($p=<0.01$ in all instances, Fisher's exact test), (Table 7-2).

Table 7-2 Summary of the Streptococcal serology data in Tourette's syndrome

Group	Mean ASOT IU/mL	Confidence intervals	Raised ASOT >200 IU/mL (%)
Child TS	299*	262-335	36/56 (64%)**
Child neurological disease	151	101-201	9/50 (18%)
Child streptococcal infection	349	270-427	45/56 (80%) n/s
Adult TS	298*	243-353	30/44 (68%)**
Adult neurological disease	140	92-188	6/50 (12%)
Adult normal controls	122	101-143	4/50 (8%)

*p=<0.01; Wilcoxon non-parametric test, ** p=<0.01; Fisher's exact test (2 tailed). Child and adult Tourette's compared with controls

Figure 7-5 Anti-streptolysin O titres in Tourette's syndrome and controls



p=<0.01; Wilcoxon non-parametric test. Child and adult Tourette's compared with controls

7.13.3 Clinical comparisons between TS patients positive and negative with ABGA

The total TS cohort had a mean diagnostic confidence index of 64% (SD 20%, range 14-100%), (Robertson *et al*, 1999). The prevalence of OCD and ADHD in the total cohort was 31% and 43% respectively, (Table 7-3). The paediatric TS patients had a mean diagnostic confidence index of 64% (SD 21%, range 14-100%), 17% incidence of OCD and a 49% incidence of ADHD. The adult TS patients had a mean diagnostic confidence index of 63% (SD 20%, range 16-100%), 51% incidence of OCD and a 31% incidence of paediatric ADHD (Table 7-3). Analysis of the ASOT titres between ABGA positive and negative TS found that 91% (21/23) of TS patients with positive ABGA Western immunoblotting had raised ASOT which was greater compared to TS patients with negative ABGA Western immunoblotting 57% (44/77), ($p<0.01$).

Table 7-3 Clinical comparisons in symptomatology between ABGA positive and negative TS patients

Result	ABGA positive (n=24)	ABGA negative (n=77)	P value
Age of first tic (range)	6.56 (range 2-13)	6.81 (range 2-16)	NS
Diagnostic confidence index mean (SD, range)	62% (SD 20, range 36-96)	64% (SD 21, range 14-100)	NS
Obsessive-compulsive behaviour/disorder %	27%	33%	NS
Attention deficit hyperactivity disorder %	22%	50%	<0.05
Family history of neuropsychiatric disease in 1st degree family member *	69.6%	75.3%	NS
ASOT >200 IU/mL	91.3%	58.4%	<0.01

P<0.01; Fisher's exact test (2 tailed).*Family history of neuropsychiatric disease includes tic disorders, Tourette's syndrome and obsessive-compulsive disorder.

7.13.4 Follow-up studies in Tourette's syndrome

Introduction

Due to a possible relationship between ABGA positivity and ASOT, patients with positive ASOT but negative ABGA were re-tested (Table 7-4). Six of the TS patients with a negative ABGA and positive ASOT in the cross-sectional study gave permission for repeat testing to see if there was a longitudinal relationship between ASOT and ABGA. The mean time for follow-up testing was 3.5 months after first testing. ASOT reverted to normal in 4/7 cases but ABGA had become positive in 5/7 cases (Table 7-4).

Table 7-4 longitudinal results in 7 patients with Tourette's syndrome: streptococcal serology and anti-basal ganglia antibodies

Case	Serology	1st Test	2nd Test (months after 1st visit)
1	ASOT	500 IU/mL	133 IU/mL
	ABGA	Negative	60 kDa (3/12)
2	ASOT	260 IU/mL	185 IU/mL
	ABGA	Negative	45 & 60 kDa (3.5/12)
3	ASOT	710 IU/mL	220 IU/mL
	ABGA	Negative	40 kDa (4/12)

Case	Serology	1st Test	2nd Test (months after 1st visit)
4	ASOT	315 IU/mL	<50 IU/mL
	ABGA	Negative	40 & 95kDa (3/12)
5	ASOT	980 IU/mL	180 IU/mL
	ABGA	Negative	45 & 60 kDa (4/12)
6	ASOT	250 IU/mL	220 IU/mL
	ABGA	Negative	Negative (3.6/12)
7	ASOT	300 IU/mL	1000 IU/mL
	ABGA	40, 45,60 kDa	40,45,60 kDa (3.8/12)

7.14 Summary of streptococcal serology results

This is the summary of all the streptococcal serology results

Table 7-5 Streptococcal serology results

Group	Number	Median ASOT (% >200 IU/mL)	Mean DNAse B (% >300 IU/mL)
Acute SC	40	349 IU/mL* (75%**)	372 IU/mL* (65%**)
Persistent SC	24	188 IU/mL (29%)	200 IU/mL (36%)
PANDAS	16	505 IU/mL* (94%**)	580 IU/mL* (81%**)
TS	56	299 IU/mL* (64%**)	Not tested
Normal control	40	97 IU/mL (16%)	92 IU/mL (10%)
Neurological controls	50	151 IU/mL (18%)	213 IU/mL (10%)
Rheumatic fever	40	300 IU/mL (72%)	350 IU/mL (60%)
Streptococcal infection	40	349 IU/mL (80%)	422 IU/mL (85%)
Streptococcal autoimmune disease	44	480 IU/mL (75%)	621 IU/mL (66%)

*p=<0.01, Wilcoxon rank-sum test (Mann-Whitney), Acute SC, PANDAS and TS versus normal and neurological controls

**p=<0.01, Fisher's exact test (2 tailed), Acute SC, PANDAS and TS versus normal and neurological controls.

7.15 Potential cross-reactivity of anti-basal ganglia antibodies with streptococcus isolated from a patient with Sydenham's chorea

Introduction

GABHS was cultured from 1 patient with acute SC (2 days post symptoms) and used to investigate cross-reactive antibody response.

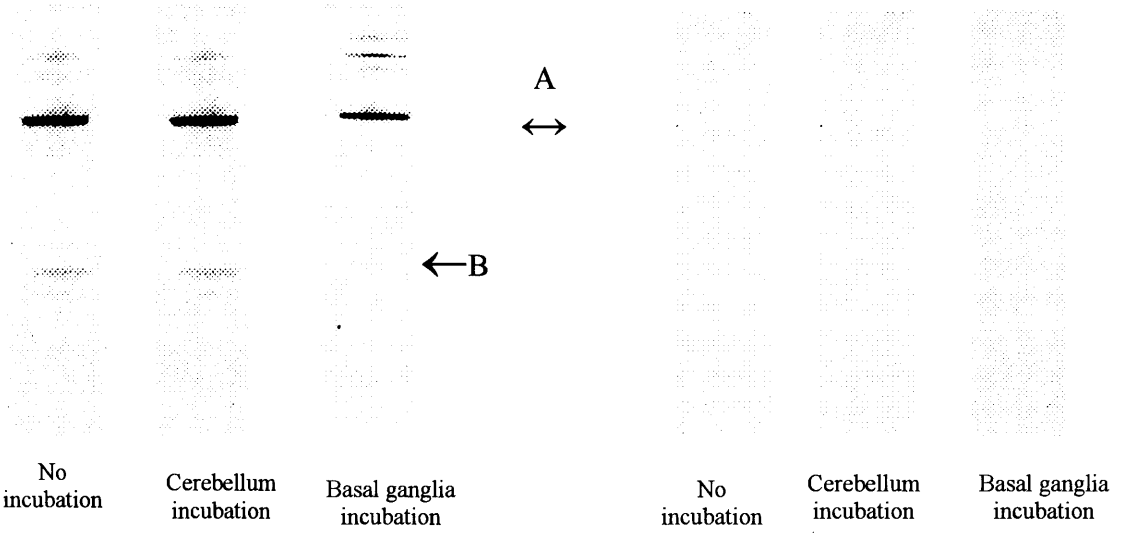
As strain specificity may and probably does influence antibody production, reactivity of IgG against brain might be different depending on strain. Therefore absorbing ABGA with streptococcus was not performed. Once, autoantigen has been found absorption experiments can be investigated.

Results

There was 1 band of IgG reactivity against both the M1 streptococcal homogenate (from the throat culture of the SC patient) and the M5 control homogenate (from a patient with PSGN), (Figure 7-6). This probably reflects the normal immune response (antibody) against streptococcal infection. In addition 2 bands were present in the M1 streptococcal homogenate that were absent from the control M5 serotype (Figure 7-6). This antibody reactivity to specific antigens was not removed by incubating serum with a human cerebellum homogenate, indicating that the antibody did not cross-react with cerebellum proteins. This antibody reactivity was totally removed by incubating the ABGA positive sera with the same concentrate of antigen but using the human basal ganglia homogenate used to detect the ABGA response (Figure 7-6). This supports cross-reactivity between the ABGA response, streptococcus and basal ganglia. The original report of the presence

of ABGA also showed that reactivity could be removed by incubating with streptococcal membranes (Rheumatogenic strains), (Husby *et al.*, 1976).

Figure 7-6 Western blot of serum tested against streptococcus M1 serotype from one patient with Sydenham's chorea, and the same serum tested against a M5 serotype from a separate patient with nephritis



A: One band of IgG reactivity which appears similar in both M1 and M5 subtypes; B: Two bands reactive against M1 subtype removed by incubating with basal ganglia homogenate

Conclusions

ASOT and anti-DNAse B results showed that serological evidence of persistent or recent streptococcal infection was common in acute SC. Unfortunately throat culture was not available in most cases so microbiological evidence of current infection could not be demonstrated. This is a major criticism of this thesis.

The high levels of antibodies against these streptococcal proteins could suggest high levels of toxin production from streptococcal infection in SC, although there is no evidence for this. In common with the ABGA findings, the presence of positive streptococcal serology was less pronounced in cases of persistent SC. This may mean that there are 2 populations within the persistent SC group. The first one showing evidence of recent or recurrent streptococcal infection with the presence of ABGA, whilst evidence for this in the other group is less persuasive.

Unfortunately only one acute GABHS culture was obtained for ABGA absorption experiments and although this did show that the antibody reactivity was associated with both basal ganglia and GABHS is not sufficient to prove this hypothesis. This experiment was only able to replicate the work performed by Husby *et al*, so further work on absorption and cross-reactivity is required.

Surprisingly the mean ASOT was higher in both adult and child TS compared to any control group except patients with uncomplicated streptococcal infection. From this data one would have expected a higher number of TS patients to be ABGA positive. This may reflect a lack of sensitivity in the existing ABGA assays or there may be an interval

between acute streptococcal infection and the appearance of ABGA. Alternatively, the presence of high titres of antibody against a streptococcal toxin may imply that the toxin plays a role in the pathogenesis of these disorders rather than ABGA themselves.

This question can only be answered by investigating streptococcal serology, levels of streptococcal toxins such as streptolysin O and ABGA over time, and comparing them to the waxing and waning of symptoms in TS. Some evidence for an association between ABGA and ASOT was seen in the TS patients who agreed to take part in a follow-up study. This showed, albeit in a small number of patients, that some patients with high ASOT but negative ABGA became ABGA positive over time. Whether this is a phenomenon common to TS or just a small subgroup will need to be answered by a longiditudinal study.

In the adult movement disorder patients studied there was no evidence for ABGA and raised ASOT in the genetic movement disorders and blepharospasm (possible autoimmune-associated). There was evidence of both ABGA and raised ASOT in patients with idiopathic dystonia and "Tourettism". This may support post-streptococcal autoimmunity in a wider spectrum of movement disorders than just chorea and tics.

8 Isolation and identification of a basal ganglia candidate autoantigen

Introduction

Despite the fact that ABGA, or more properly anti-neuronal antibodies with axonal, cytoplasmic staining are of low concentration. Candidate antigens from the S1 fraction of human basal ganglia were identified using proteomics. This was a major aim of this thesis.

8.1 Identification of a basal ganglia candidate autoantigen

Serum samples which reacted against a single putative autoantigen were studied where possible. There were several difficulties in employing a proteomic methodology, because patient serum contains polyspecific antibodies and only a small percentage of the immunoglobulin was specific to any of the target autoantigens. The ABGA response is also of low concentration compared to other neuronal antibodies. Availability of basal ganglia tissue to conduct large scale separation and identification of candidate autoantigens was also a limiting factor in these studies. Reproducibility of the electrophoretic methods was also a problem.

8.2 Results of the immunoprecipitation method

The samples were subjected to PAGE and silver stain but bands were only visible in the first elute collected from the spin-cup. Western immunoblotting of this elute coupled with probing of the electro-transferred protein with ABGA positive serums resulted in weak reactivity to 1 band. Mass spectroscopy of this band identified contaminating albumin and there was insufficient protein for identification of the other protein. The immunoprecipitation was repeated using the maximum concentration of antibody and antigen recommended (Perbio immunoprecipitation kit) with an increased number of washes to remove contaminants. The precipitated proteins were weakly reactive on Western blot with ABGA positive sera. A gel was also silver stained and the proteins were cut from the gel but, again the protein could not be identified by mass-spectroscopy because of low protein concentration.

Conclusion

To overcome the methodological problems the proteins recovered from the immunoprecipitation were concentrated, using a centrifugal filter concentrator (Millipore). Unfortunately mass-spectroscopy was still unable to identify the protein due to low concentration. The proteins were not able to be concentrated further as this resulted in insufficient volume for mass-spectroscopy.

The immunoprecipitation method was not investigated further proteomics (2-D electrophoresis and mass spectroscopy) were investigated.

8.3 Identification of neuron-specific enolase as a candidate autoantigen in Sydenham's chorea using 2-D electrophoresis and proteomics

Introduction

Two-dimensional electrophoresis was carried out using an 18cm long immobiline, pH 3-10, gel strips (Amersham Pharmacia) with silver staining of the second dimension PAGE gel.

Results

This resulted in several hundred protein 'spots'. However, the acidic and basic areas of the gels contained some overlapping areas rather than discrete proteins. The proteins were transferred to nitrocellulose and probed with ABGA (40, 45, 60 or 98 kDa) positive sera. This resulted in spots in the acidic area of the gel when the proteins were Western blotted. Neurone-specific enolase was identified but resolution was poor, method required large amounts of protein, contaminating albumin, haemoglobin and IgG fragments were all methodological problems.

Conclusion

This method was abandoned and a pI 3-10 range IEF gel mini system (Invitrogen) was used to identify candidate antigens throughout.

This does not mean that other membranous autoantigens might be important in Sydenham's chorea, the soluble antigens are still important as they might be a diagnostic marker of disease. This has implications for diagnostics and treatment so thus is important.

8.4 Two-dimensional electrophoresis of human basal ganglia S1 (soluble) fraction

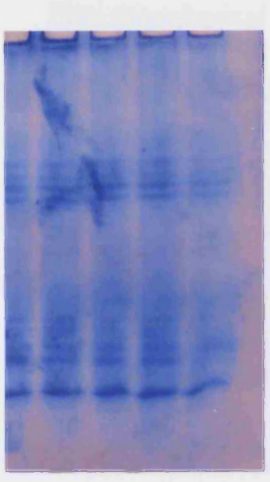
Introduction

The S1 fraction was prepared as detailed in the methods section. An equal volume of homogenate was added to sample buffer (Invitrogen), mixed for 30 mins and subjected to 2-D using a pH 3-10 gel (Invitrogen). The gel was silver stained (Amersham-Pharmacia).

Results

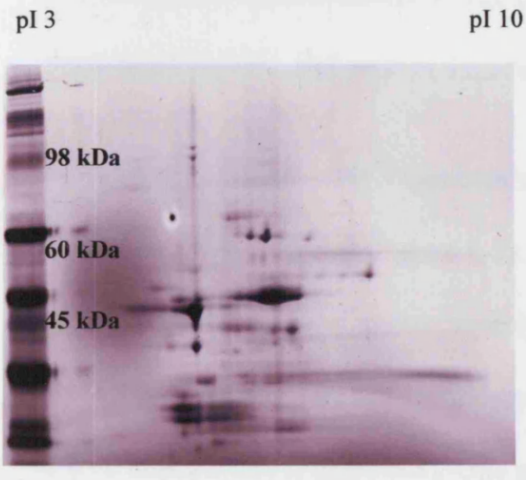
A typical stained IEF gel of human basal ganglia (soluble S1 fraction) is shown in figure 8-1. The soluble S1 cytosolic fraction was used to identify putative antigens by 2-D electrophoresis as this had the most antibody reactivity in SC, PANDAS and TS. A stained 2-D gel is shown in Figure 8-2.

Figure 8-1 Commassie stained isoelectric focussed pH 3-10 gel of soluble S1 fraction of human basal ganglia for second dimension PAGE incorporating 2-D electrophoresis



Gel is only stained for 5 minutes with commassie as per manufactures Instruction (Invitrogen), therefore proteins are only weakly stained

Figure 8-2 Typical silver stained 2-D electrophoretic separation of soluble basal ganglia fraction



8.5 Reactivity to basal ganglia proteins in Sydenham's chorea and controls

Introduction

Five neurological disease controls and 5 SC patients were tested against Western blots of 2-D electrophoretic gels.

8.6 2-D electrophoresis using five Sydenham's chorea and five normal sera

Introduction

Five patients with Sydenham's chorea who had 40, 45, 60 or 98 kDa antibody responses against soluble S1 basal ganglia fraction were tested and shown in figures 8-3 and 8-4.

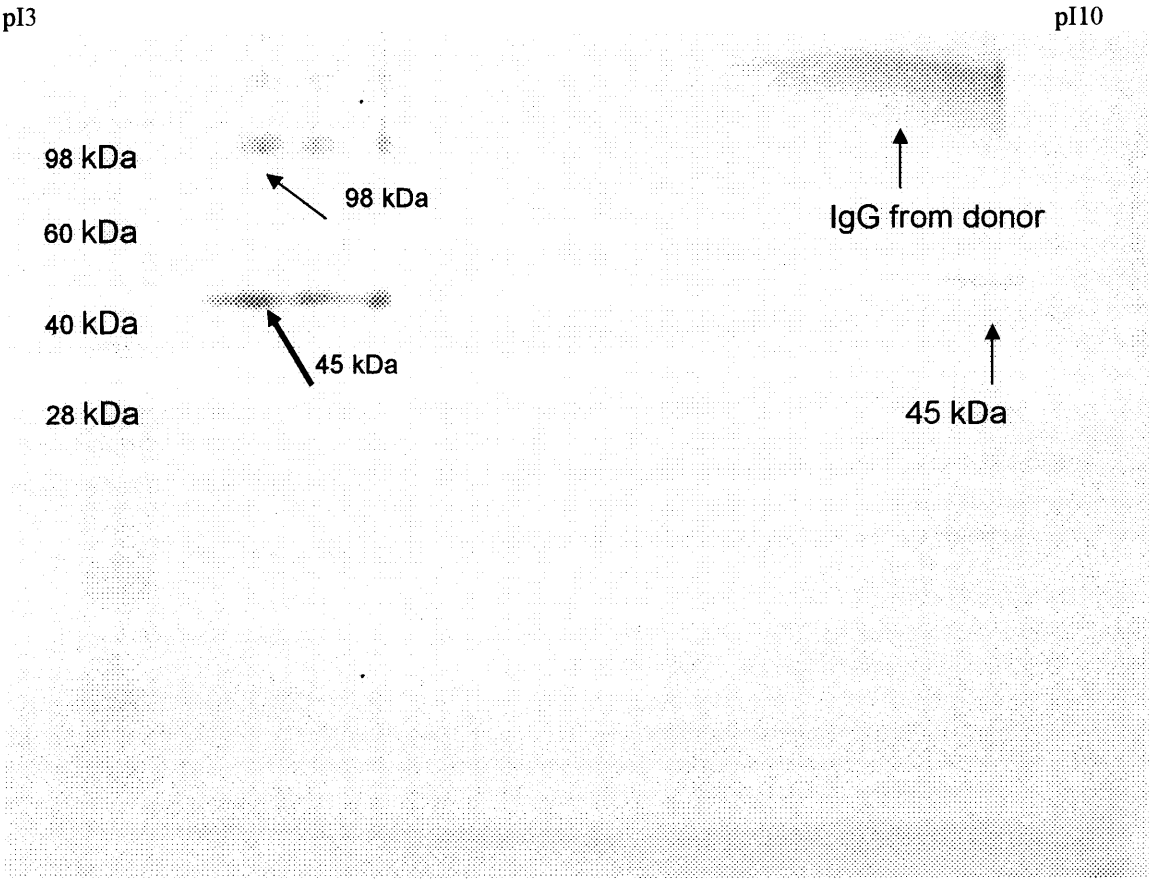
Results

Comparison of the staining using ABGA or samples from controls showed that the 98 and 45 kDa (Figure 8-2 and 8-3) proteins were localised at the acidic end of the IEF gel and had a pI of approximately 4. The proteins were cut out of the gel and subjected to mass-spectroscopy, and protein data base search.

Serum from a patient with Sydenham's chorea bound to the 45 and 98 kDa proteins with a pI of approximately 4.0 (Figure 8-3). Additionally the serum also recognised a 45 kDa protein which has a pI of approximately 7.0 (Figures 8-3). A streak of IgG from the donor tissue was recognised by the secondary antibody (Figure 8-3). There is also trace binding to a 60 kDa protein (Figure 8-3). One patient also had weak binding to a 40 kDa protein (Figure 8-4). This could not be determined by mass-spectroscopy due to contamination with other proteins.

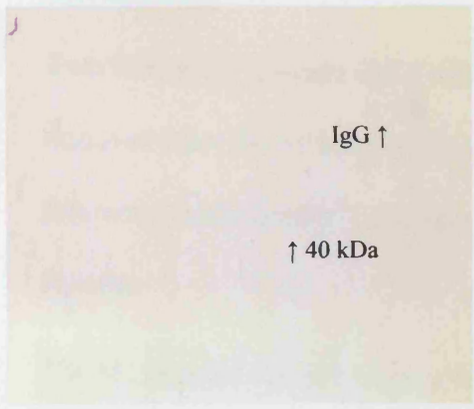
Four of the 5 SC patients had binding to a 45 kDa acidic protein, 1 had binding just to the basic protein and 1 had binding to both. Three patients with SC also had binding to a 98 kDa band. None of the controls had any binding.

Figure 8-3 Serum from one patient with Sydenham's chorea diluted 1/300 and tested against a Western immunoblot of a 2-D electrophoretic separation of soluble basal ganglia fraction to confirm specificity of serum staining and molecular weight of proteins which were identified by mass spectroscopy



Serum diluted 1/300 recognised two proteins with a pI of approximately 4.0. There was weak binding to a 45 kDa protein of pI 7.0. IgG from the donor was also recognised. All proteins were identified by mass spectrometry by Dr. R Wait, PhD.

Figure 8-4 One patient with Sydenham's chorea with basic 40 kDa antibodies and absent acidic 45 kDa



Conclusions

The 45 and 98 kDa bands could be detected using 2-D electrophoresis of a pH 3-10 gel (Invitrogen). None of the controls had binding to these proteins. Contaminating IgG was a problem, therefore human soluble S1 fraction of basal ganglia was incubated with anti-human IgG coated agarose beads to reduce this contamination. Additional SC samples were tested as well as these detailed in this experiment.

8.7 Two-dimensional electrophoresis with donor IgG removed by agarose beads incubated with 2 Sydenham's chorea samples and 1 normal control diluted 1/300

Introduction

Two further SC patients and 1 normal control were tested at 1/300. Donor IgG was removed from the S1 fraction by incubating with agarose coated anti-IgG beads following manufacturer's guidelines (Sigma). Results are shown in figure 8-5.

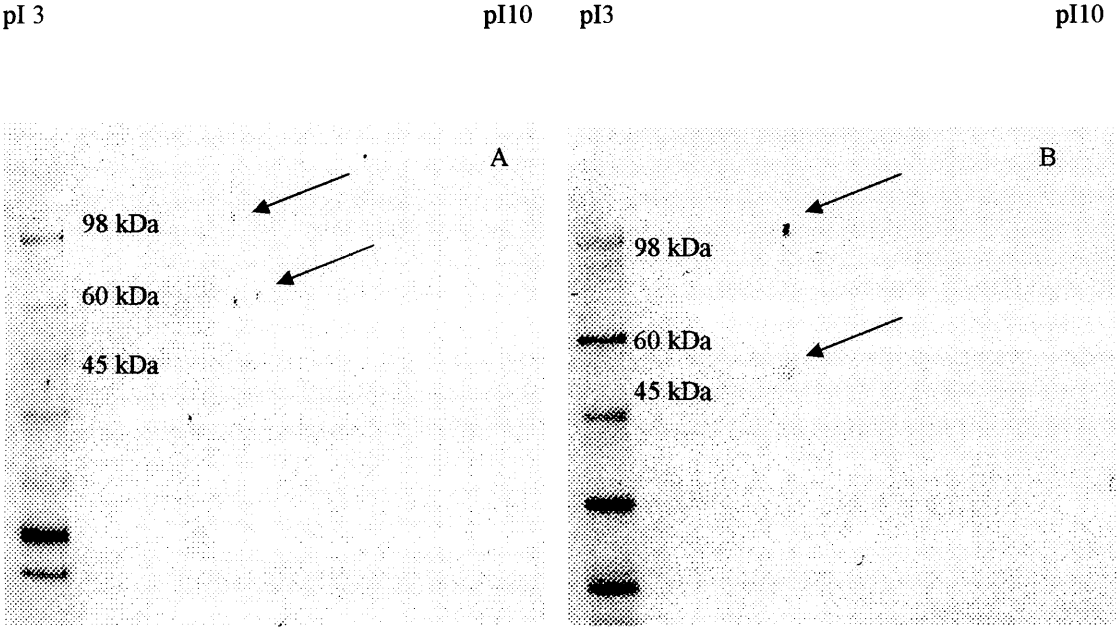
Results

The SC samples tested bound to the same antigens as seen in Western blotting with 45, 60 and 98 kDa bands being the most common (Figure 8-5). Normal controls did not bind to any protein (Figure 8-5).

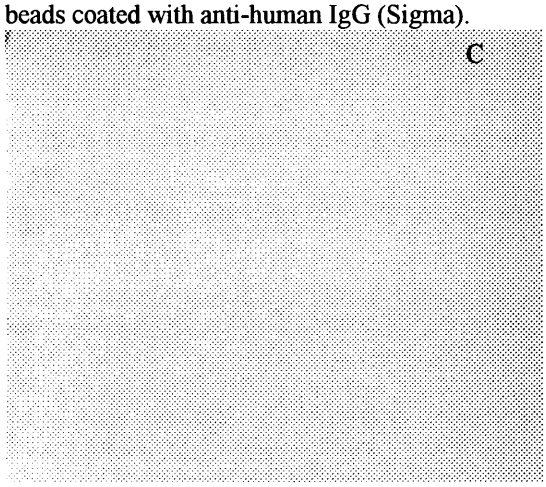
Conclusion

The 45, 60 and 98 kDa protein spots were subjected to mass-spectroscopy. The 40 kDa band did not appear to be present. Subsequently the 40 kDa band was identified as a basic protein using immunoprecipitation and 2-D electrophoresis by Dr. Russell Dale (Dale et al., 2006) which could explain why I couldn't get reactivity to this protein on my gels.

Figure 8-5 Two Sydenham's chorea anti-basal ganglia positive sera tested against 2 Western immunoblots of 2-D electrophoretic separation of soluble basal ganglia with IgG from donor removed



A: SC patient diluted 1/300, IgG reactivity to 98 kDa proteins which were excised from the gel at the same molecular weight points; B: SC patients with 45 kDa reactivity and possible 60 kDa reactivity. No reactivity to IgG or non-neuronal enolase. IgG was removed by incubating homogenates with agarose beads coated with anti-human IgG (Sigma).



C: Normal control diluted 1/300 and tested against the same S1 fraction, no reactivity against any protein

8.8 Neuron-specific enolase is a possible autoantigen in Sydenham's chorea

The 45 and 98 kDa proteins were cut from the gel and the amino acid sequence were identified by MALDI-TOF mass-spectroscopy. This was kindly carried out by Dr Robin Wait, PhD, Kennedy Institute of Rheumatology, Imperial College London. The proteins were identified using Entrez- protein data base (www.ncbi.nlm.nih.gov).

Results

The complete amino acid sequence of the protein identified by mass-spectroscopy is in the shaded area (Figure 8-6). The proteins of pI 4 were gamma, neuronal specific enolase, a glycolytic enzyme (Figure 8-6). The 45 kDa band was the monomer and the 98 kDa band the dimer. The 45 kDa protein of pI 7.0 was non-neuronal alpha enolase a ubiquitous enzyme. The highlighted sequences are individual ion scores >51 indicate identity or extensive homology. The summed Mascot ion score for the gamma enolase recognised was 500, (p=<0.05), (Dale *et al.*, 2006). The pI of alpha enolase is reported to be 7.0 and gamma 4.0 (www.ncbi.nlm.nih.gov).

Figure 8-6 The amino acid sequence of Homo-sapien gamma enolase with the shaded areas showing sequences found in the protein 'spots' excised from the gel

```
  1 msiekiware ildsrgnptv evdlytakgl fraavpsgas tgiyealelr dgdkqrylgk
 61 gvlkavdhin stiapaliss glsvveqekl dnlmleldgt enkskfgana ilgvslavck
121 agaaerelpl yrhiaqlagn sdlilpvpaf nvinggshag nklamqefmi lpvgaesfrd
181 amrlgaevyh tlkgvikdky gkdatnvgde ggfapnilen sealelvkea idkagyteki
241 vigmdvaase fyrdgkydld fksptdpsry itgdqlgaly qdfvrdypvv siedpfdqdd
301 waawskftan vgiqivgddl tvtnpkrier aveekacncl llkvnqigsv teaiqackla
361 qengwgvmvs hrsgetedtf iadlvvglct gqiktgapcr serlakynql mrieeelgde
421 arfaghnfrn psvl
```

The molecular weight of the enolase protein cut from the gel (45 kDa candidate) was 47kDa and had 57.8% coverage of human gamma enolase. The other identity of this protein was gamma enolase but from different species and alpha enolase. The 98 kDa candidate was the dimeric form of the same protein

8.9 Confirmation of enolase as a candidate antigen

Introduction

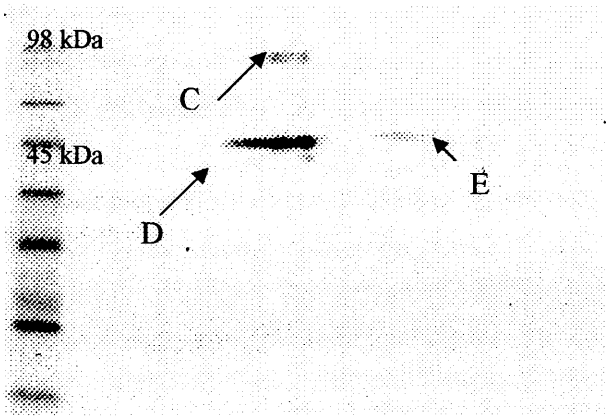
To confirm the identity of enolase a commercial, polyclonal anti-enolase antibody (C-19) (Figure 8-7) and monoclonal NSE (P-2) were purchased (Autogen Bioclear, UK).

8.9.1 2-D electrophoresis using commercial antibodies

Introduction

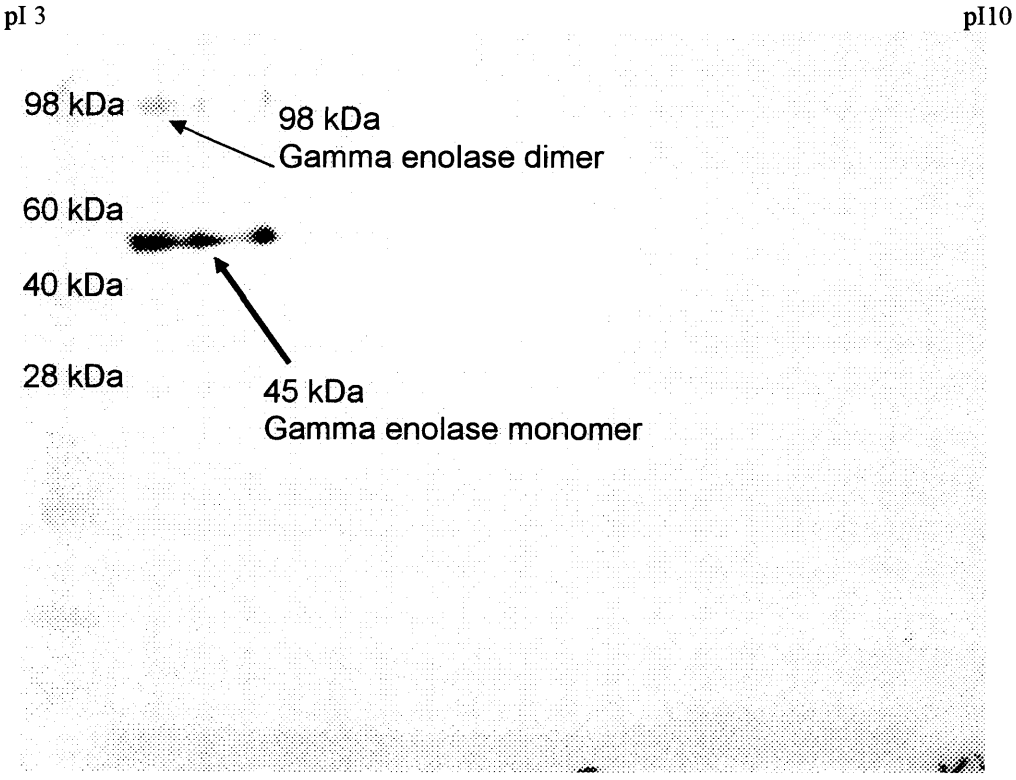
The commercial antibodies were used to probe the same basal ganglia homogenate separated by 2-D electrophoresis and blotted onto nitrocellulose (Figure 8-7 and 8-8).

Figure 8-7 Commercial polyclonal anti-enolase antibody c-19 tested against a Western immunoblot of a 2-D electrophoretic separation of soluble basal ganglia fraction to confirm specificity of serum staining and molecular weight of protein



Commercial anti-enolase antibody (C-19) at 1:2000 dilutions with colorimetric development; C and D: Binding to the dimeric (98 kDa) and monomeric (45 kDa) forms of NSE; E: non-neuronal enolase (45 kDa) which has a pI of approximately 7.0.

Figure 8-8 Commercial monoclonal P-2 anti-neurone specific enolase antibody NSE tested against a Western immunoblot of a 2-D electrophoretic separation of soluble basal ganglia fraction to confirm specificity of serum staining and molecular weight of proteins



Neuron specific enolase monoclonal antibody recognises two proteins with a Pi of approximately 4.0. The monoclonal antibody recognises the monomer 45 kDa and dimer 98 kDa of the enolase protein. No binding to the non-neuronal enolase.

Results The pattern of reactivity (protein 'spots') and molecular weights was similar to that seen with 98 kDa and 45 kDa ABGA positive SC serums. The commercial polyclonal antibody also recognised non-neuronal specific enolase which had a pI of

approximately 7, whilst (gamma, gamma) NSE was identified as a protein with a pI of approximately 4.0.

8.10 Homology between gamma and streptococcal enolase

Streptococcal species (including GABHS) have enolase on their cell surface as well as cytosolic (Fontan *et al.*, 2000). This could support the hypothesis of molecular mimicry induced autoantibody production in SC and PANDAS. The structure of GABHS and human neuron specific enolase is presented in figure 8-9.

Figure 8-9 Streptococcus pyogenes enolase

Streptococcus pyogenes enolase

```
  1 msiitdvyar evldsrgnpt levevytesg afgrgmvpsg astgeheave lrdgdksryl
 61 glgtqkavdn vnniiaeaii gydvrdqqai dramialdgt pnkgklgana ilgvsiavar
121 aaadylevpl ytylggfntk vlptpmmnii nggshsdapi afqefmimpv gaptfkeglr
181 wgaevfhalk kilkerglvt avgdeggfap kfegtedgve tilkaieaag yeagengimi
241 gfdcassefy dkerkvydyt kfegegaavr tsaeqvdyle elvnkypiit iedgmdendw
301 dgwkvlterl gkrvqlvgdd ffvtnteyla rgikenaans ilikvnqigt ltetfeaiem
361 akeagytavv shrsgeteds tiadiavatn agqiktgsls rtdriakynq llriedqlge
421 vaqykgiksf ynlkk
```

Human neuron specific enolase

```
  1 msiekiware ildsrgnptv evdlytakgl fraavpsgas tgiyealelr dgdkqrylgk
 61 gvlkavdhin stiapaliss glsvveqekl dnlmleldgt enkskfgana ilgvslavck
121 agaaerelpl yrhiaqlagn sdlilpvpaf nvinggshag nklamqefmi lpvgaesfrd
181 amrlgaevyh tlkgvikdky gkdatnvgde ggfapnilen sealelvkea idkagyteki
241 vigmdvaase fyrdgkydld fksptdpsry itgdqlgaly qdfvrdypvv siedpfdqdd
301 waawskftan vgiqivgddl tvtnpkrier aveekacncl llkvnqigsv teaiqackla
361 qengwgvmvs hrsgetedtf iadlvvglct gqiktgapcr serlakynql mrieeelgde
421 arfaghnfrn psvl
```

Results

A protein-protein blast sequence homology analysis was performed to investigate homology between these proteins using http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2 data base. There was 68% homology of amino acid sequence between the human and streptococcal enolase (score = 398 bits (1022), Expect = e-109 Identities = 211/430 (49%), Positives = 294/430 (68%), Gaps = 14/430 (3%) were obtained), (Appendix 12.7).

Therefore identical sequence identities were: 49% between human and GABHS enolase with expected chance identities being 10^{-109}. A list of homologous sequences is presented in appendix 12.7. The longest homologous sequence was: VSHRSGETED and is highlighted in both GABHS and human enolase sequences in Figure 8-9

Conclusion

Neuron specific enolase is a putative autoantigen in Sydenham' s chorea, although as enolase is brain specific rather than basal ganglia, anti-basal ganglia antibodies are not specific to the basal ganglia. Homology with a streptococcal protein exists which does support the original hypothesis of molecular mimicry.

As NSE is considered to be cytoplasmic a functional effect of antibody binding is unlikely. Indeed antibodies against NSE might just be due to GABHS infection, are transient, and of no consequence. Sydenham's chorea and normal control IgG responses to GABHS might help to examine the role of enolase antibodies further.

8.11 Two-dimensional electrophoresis of a Streptococcus isolated from a patient with Sydenham's probed with the commercial polyclonal anti-enolase

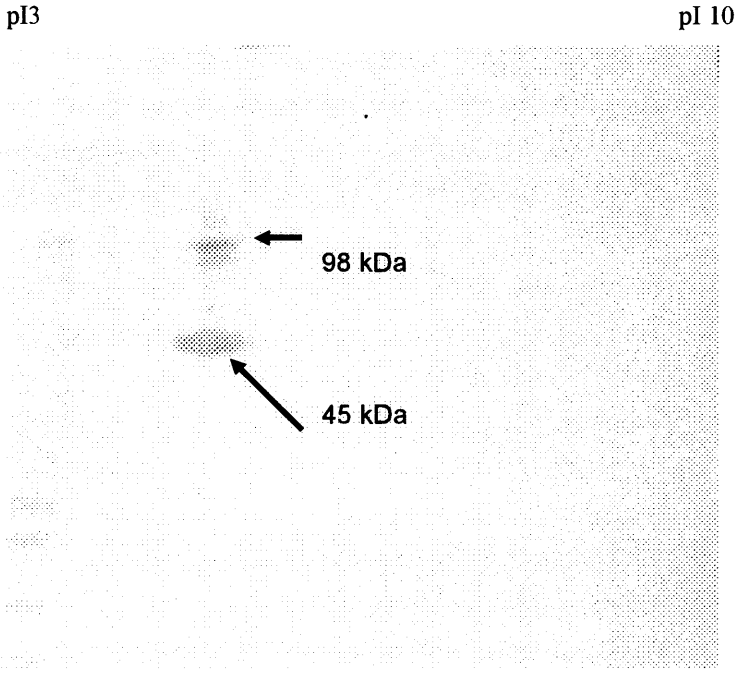
Introduction

A GABHS strain isolated from a patient with SC was homogenised and subjected to 2-D electrophoresis to investigate and compare (molecular weight and pI) enolase from GABHS (Figure 8-10). The gel was probed with same antibody used for basal ganglia.

Results

Streptococcal enolase has the same molecular weight and pI on the basis of this experiment.

Figure 8-10 2-D electrophoresis of group A streptococcus probed with polyclonal anti-enolase antibody C-19



Commercial polyclonal enolase diluted 1/1000 identified 2 bands of molecular weights 45 and 98 kDa with a pI of 4. There was no reactivity with a basic isoforms of enolase unlike human basal ganglia

8.12 Reactivity of IgG from Sydenham's chorea and controls against a 2-D electrophoresis of Streptococcus

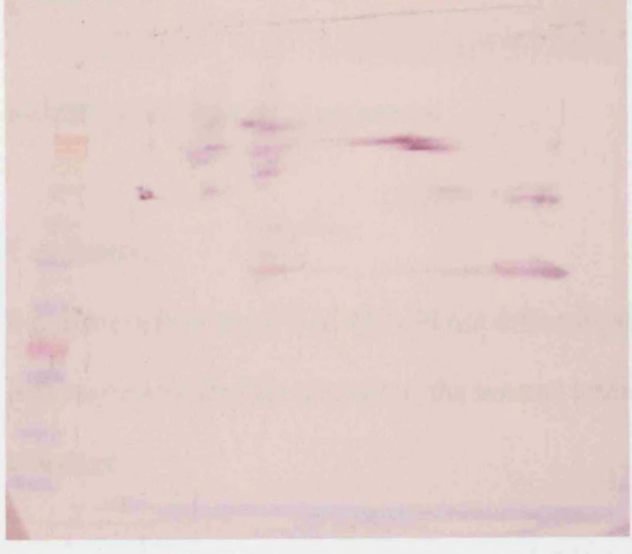
Introduction

To assess cross-reactivity of IgG anti-basal ganglia antibodies and GABHS proteins, streptococcus from a patient with SC was subjected to 2-D electrophoresis and 5 controls and SC patients (Figure 8-11 and 8-12) were tested.

Results in normal controls

There were multiple bands of reactivity in normal controls which shows the polyspecific nature of the IgG response against streptococcus.

Figure 8-11 2-D electrophoresis gel probed with 1/500 diluted serum from a normal (non-diseased) control

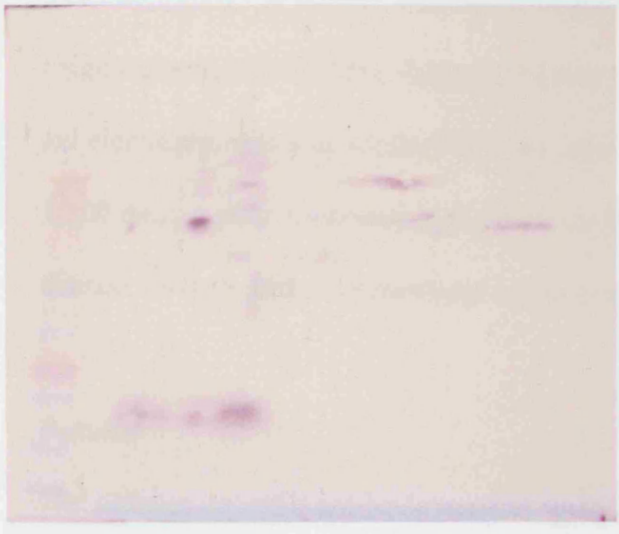


Multiple spots of reactivity was present

Results in Sydenham's chorea

There were multiple bands of reactivity in SC patients which shows the polyspecific nature of the IgG response against streptococcus.

Figure 8-12 2-D electrophoresis gel probed with 1/500 diluted serum from a Sydenham's chorea patient



Multiple spots of reactivity are present

Conclusion

2-D electrophoresis of GABHS did not differentiate SC and controls. This may mean that anti-enolase antibodies are part of the normal immune response against GABHS infection.

However, this method does not differentiate anti-enolase antibody reactivity from background binding to other GABHS antigen. A purified or recombinant antigen source is required. The identification of NSE as a putative antigen in post-streptococcal movement disorders has been published (Dale *et al.*, 2006).

8.13 Anti-Neurone specific enolase antibodies in Sydenham's chorea and controls using Western Immunoblotting of human enolase antigen from a commercial source

Introduction

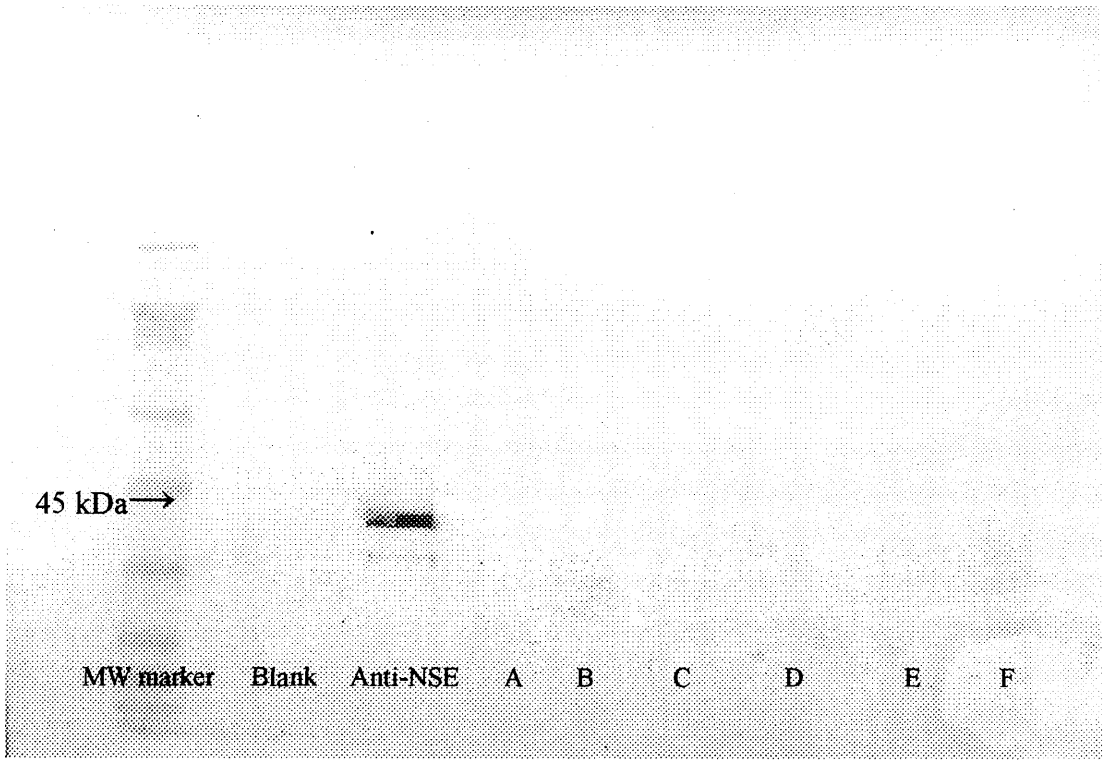
Ten micrograms of human Neurone specific enolase (NSE) (90% purity) was purchased (Sigma chemicals, N4773). One microgram per blot was subjected to a 4-12% NuPage gel electrophoresis and Western blotted onto nitrocellulose. Lane 1 was probed with 1/500 diluted polyclonal anti-NSE antibody (C-19, Autogen Bioclear). Six neurological disease controls and six Sydenham's chorea samples were tested.

Results

The commercial NSE antibody reacted against a 45 kDa band. Samples from patients with neurological disease were also tested against this blot but none of them had reactivity against the NSE band (Figure 8-13).

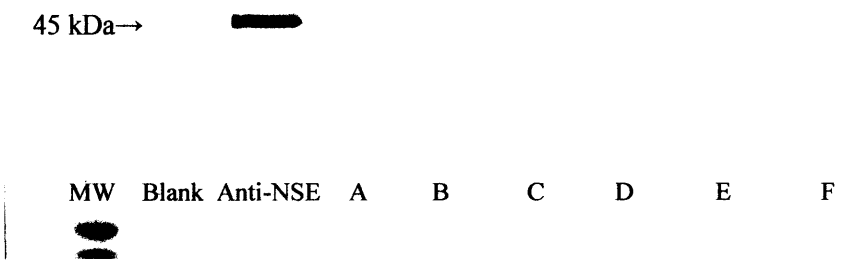
Sixty-six percent of patients with acute SC (4/6) had a definite band which aligned with that identified by the anti-NSE commercial antibody. Seventeen percent of patients with SC were negative (1/6) and 1 patient had only weak reactivity (Figure 8-14). The weakness of this experiment is the small number of patients tested but the expense of commercial antigen is prohibitive to large experiments. Therefore an alternative source of antigen is required. Recombinant antigen would be the next step.

Figure 8-13 Six controls diluted 1/300 and tested against 1 microgram of neuron-specific enolase on a Western blot



Blank: Secondary detector antibody only; Anti-NSE: anti-neuron specific enolase commercial antibody; A: DYT1 positive dystonia; B: Huntington's chorea; C: Parkinson's disease; D: PSGN; E: streptococcal pharyngitis; F: multiple sclerosis.

Figure 8-14 Samples from patients acute Sydenham's chorea tested against 1 microgram of neuron-specific enolase on a Western immunoblot



MW: Molecular weight marker; Blank: Secondary detector antibody only; Anti-NSE: anti-neuron specific enolase commercial antibody; A-F: acute SC.

8.14 Recombinant enolase as a source of antigen in future research

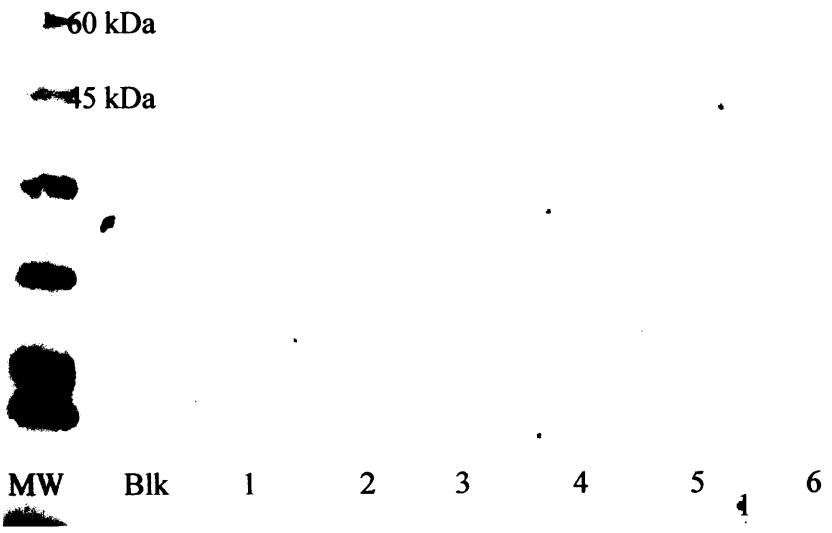
Introduction

To investigate the role of anti-enolase antibodies in post-streptococcal neurology syndromes, recombinant proteins are essential for further research. A NSE recombinant protein was provided by Mr P. Candler (Neuroinflammation, Institute of Neurology, London).

Results

Recombinant antigen gave a similar pattern of results to commercial NSE (Figure 8-15).

Figure 8-15 Recombinant gamma enolase Western immunoblot



1: Parkinson's disease; 2: commercial monoclonal ab NSE P2; 3: MS; 4: ?Adult PANDAS; 5: TS; 6: Basal ganglia stroke

8.15 Regional brain specificity of anti-enolase antibodies

As NSE is neuronal specific rather than just basal ganglia, the original hypothesis of basal ganglia specificity has been cast into doubt. However, the reactivity of NSE antibodies in SC, PANDAS and TS might have been related to antigen presentation. A Western blot of soluble human basal ganglia, spinal cord, cerebellum and heart fractions was prepared, normalised for protein concentration (3μg per lane) and one SC patient with anti-NSE antibodies was diluted 1/300 and tested against all fractions (Figure 8-16)

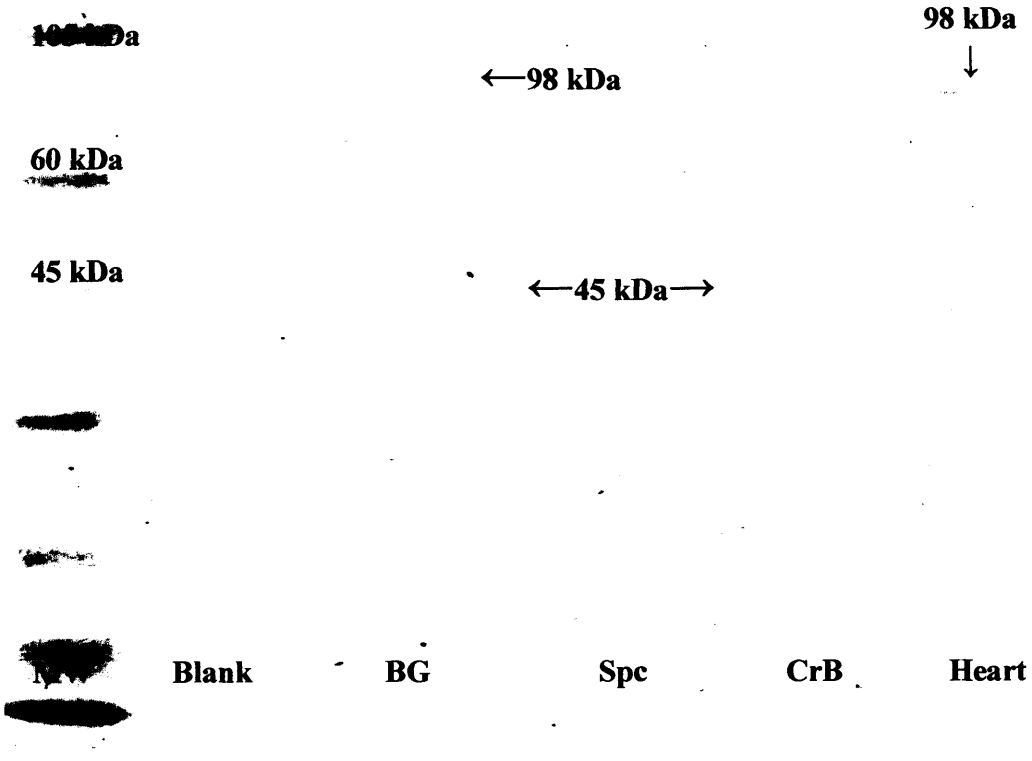
Results

The reactivity to 45 kDa enolase was present in basal ganglia and cerebellum, but not spinal cord, this band was also present in heart tissue. However, the serum from a SC patient also reacted with the 98 kDa dimer of NSE, but this reactivity was only present in the basal ganglia and heart fractions (Figure 8-16). Therefore an anti-NSE antibody directed aginst the dimer (gamma gamma) of enolase might be specific to basal ganglia and heart (Figure 8-16).

Conclusion

However, only 1 sample was tested and this is only a hypotheis that requires much further research. Identifcation of a 98 kDa enolase dimer from GABHS is also essential. A grant from the Tourette's society of America (TSA) has been awarded for this research and is being conducted in the Microbiology Department at the Institute of Child Health, Great Ormond Street Hospital. London.

Figure 8-16 One Sydenham's chorea patient with anti-enolase antibodies tested on Western blot against brain fractions and heart



MW= molecular weight marker, Blank=2nd antibody only (Rabbit anti-Hu IgG), BG=basal ganglia, Spc=Spinal cord, CrB=Cerebellum, Heart=cardiac tissue.

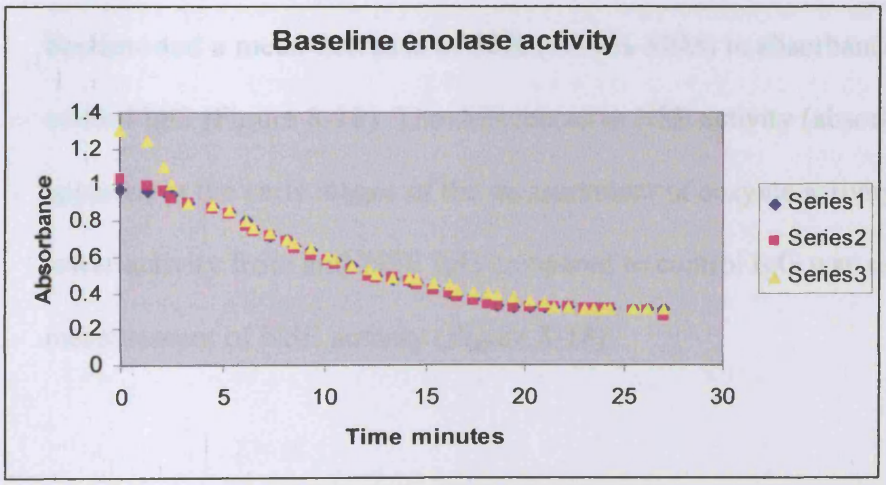
8.16 A possible functional effect of anti-enolase antibodies

To investigate whether anti-NSE antibodies might have a role in pathogenesis the effect on glycolytic function was studied in a pliot experiment by testing 1/300 diluted serum against purified rat brain enolase. The enolase was provided by Dr. Russell Dale, Institute of Child Health, Great Ormond Street Hospital. London.

8.16.1 Result of baseline activity of neuron specific enolase

Measuring the baseline glycolytic activity of NSE was found to be consistently reproducible as expected.Three experiments of normal NSE activity were performed to assess this (Figure 8-17). The standard error of the mean for this assay was no greater than 0.08 absorbance units between replicate measurements. All data points are shown in Figure 8-17.

Figure 8-17 Reproducibility of enolase activity



8.16.2 Initial effects in activity when anti-enolase antibodies were incubated with neuron specific enolase

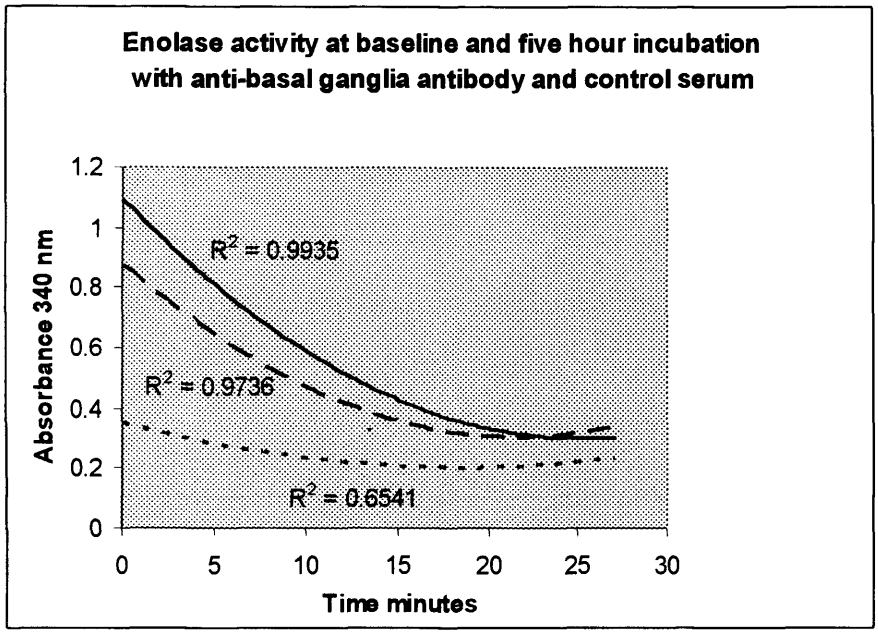
Purified IgG (Appendix) from a serum sample positive for anti-NSE antibodies and control IgG were incubated with NSE to assess changes in enzyme activity. This experiment was performed three times with no significant differences between the results of enzyme activity measured over time. There was no difference in enolase activity between control and anti-NSE IgG after 3 hours at room temperature.

8.16.3 The effect of antibody incubation on enolase activity after five hours

After 5 hour incubation of NSE with control IgG, enolase activity was slightly reduced compared to the baseline activity (Figure 8-18) however, there was a greater decrease in NSE activity in the presence of anti-NSE IgG compared to control IgG (Figure 8-18). This could not be explained by the observed reduction in enzyme activity seen with control IgG (Figure 8-18).

Anti-NSE IgG caused a mean decrease in absorbance of 48% (+/- 5% SEM) compared to baseline and a mean decrease of 58% (+/- 8% SEM) in absorbance when compared to control IgG (Figure 8-18). The differences in NSE activity (absorbances) were most apparent in the early stages of the measurement of enzyme activity (Figure 8-18). This lower activity from anti-NSE IgG compared to control IgG was seen throughout the measurement of NSE activity (Figure 8-18).

Figure 8-18 Results of a five hour incubation of NSE with anti-enolase and control IgG

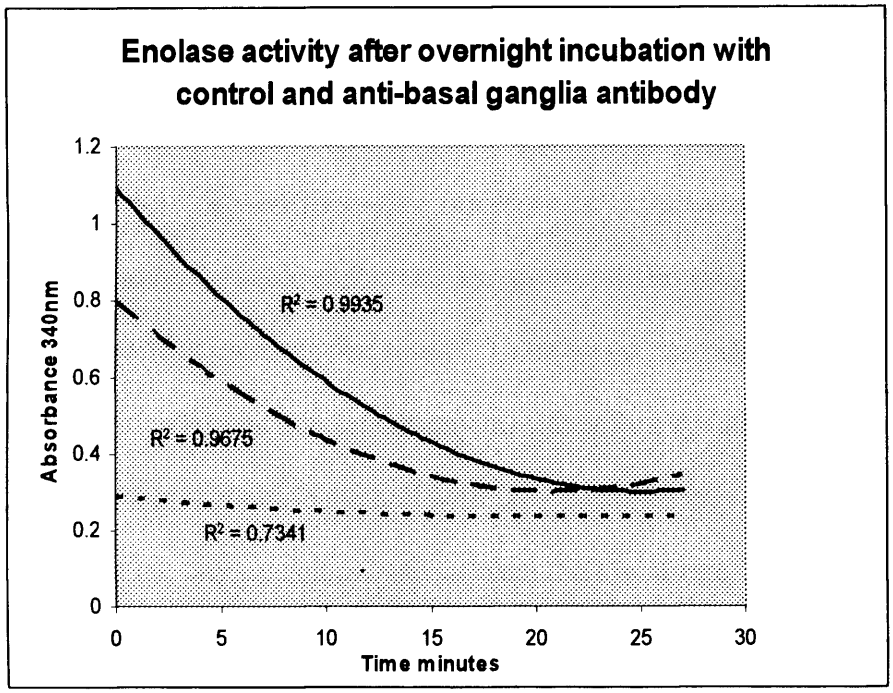


Baseline enolase activity (-), enolase activity with five hour incubation of normal control (- - - -), enolase activity with five hour incubation of ABGA (. . . .).

8.16.4 The effect of antibody incubation on enolase activity after an overnight incubation

Similarly, enolase activity with control IgG was reduced with an overnight incubation (Figure 8-19). However, there was a decrease in NSE activity incubated with anti-NSE IgG (Figure 8-19) compared to control IgG. There was a mean decrease in absorbance of 72% (+/- 10% SEM) in the activity of this NSE compared to baseline and a mean decrease in absorbance of 38% (+/- 5% SEM) compared to control IgG. These differences were most apparent in the early stages of the activity experiment but were apparent throughout.

Figure 8-19 Results of overnight incubation of enolase activity with anti-enolase and control IgG



Baseline enolase activity (-), enolase activity with five hour incubation of normal control (- - -), enolase activity with five hour incubation of ABGA (. . . .).

Conclusion

There were no significant alterations in enzyme activity with anti-enolase positive IgG. However, an overnight incubation did show a decrease, although this may reflect methodology rather than a functional effect of antibody. This experiment does not rule out an effect on ATP production of the neuronal membrane. Cell culture experiments using primary striatal cultures are a possible method to examine this further.

However, an alternative hypotheis for a functional effect of anti-enolase antibodies was reported by Dale *et al.*, 2006). Compared to anti-Hu, anti-enolase antibodies induced a significantly higher rate of apoptosis in cultured cerebellar granule cells (Dale *et al.*, 2006). Therfore an alternative functional effect of antibodies might be neuronal death.

8.17 Identification of the 40 and 60kDa putative antigens

Unfortunately, 2-D electrophoresis and proteomic investigation of the 40 and 60 kDa proteins yielded inconclusive results. There was contamination of the candidate protein 'spots' with albumen and IgG fragments (identified by mass-spectroscopy). Combining the immunoprecipitation method with 2D-electrophoresis also resulted in no significant results, as the protein concentrations were too low for mass-spectroscopy. The main problem in identification of the 40 and 60 kDa autoantigens was the availability of basal ganglia tissue for 2-D electrophoresis.

Conclusion

The identification of the 40 and 60 kDa proteins was conducted as part of a separate PhD by Dr Russell Dale, PhD. The 40 kDa protein was identified as Aldolase C and the 60 kDa protein as pyruvate kinase M1. This work was carried out by Dr Russell Dale, PhD using ammonium sulphate precipitation and Fluid Phase Liquid Chromatography (Dale *et al.*, 2006). As the antigens are enzymes in the glycolytic pathway, a possible effect of antibody binding was proposed. The results of the enolase enzyme inhibition have shown blockade of glycolysis is probably not a major feature of anti-glycolytic enzyme antibodies.

8.18 Final conclusions

It was surprising that this candidate autoantigen(s) were not basal ganglia specific as the original paper by Husby *et al* suggested it would be. However my IF studies suggested weak neuronal staining in basal ganglia, cerebellar granular layer and cortex.

Neurone- specific enolase has homology with a GABHS surface bound enolase and this does supports molecular mimicry as a hypothesis for ABGA production in SC and PANDAS. As an extension of this work we are synthesising recombinant NSE to establish an ELISA and Western immunoblotting method to test the percentage of SC, PANDAS and TS patients with anti-NSE antibodies. Purified NSE purchased from a commercial source did find anti-NSE antibodies in 4/6 patients with acute SC and no reactivity in controls.

To assess if anti-NSE antibodies could be pathogenic the effect of anti-NSE antibodies on glycolytic function was assessed. Antibodies against NSE did not have a major affect on the enzymatic function of NSE. However, the most profound effects of these antibodies appeared to be seen during the initial measurement of the enzyme activity method and overnight antibody incubation. This means that enzyme occupancy with antibody could be accumulative in its effect. The effect of anti-NSE antibodies on neuronal cell culture are experiments that are ongoing to assess whether metabolic stress could have an effect on neuronal survival and functioning.

Human neuron specific enolase was found to be a candidate autoantigen for the 45 and 98 kDa proteins. Dr Russell Dale, PhD, identified Aldolase C and Pyruvate Kinase M1 glycolytic enzymes as the other candidate antigens in post-streptococcal movement and neuropsychiatric disorders (Dale *et al.*, 2006).

This thesis adds to an antibody hypothesis of Sydenham's chorea and other movement and neuropsychiatric disorders associated with streptococcal infection. How a common infection is associated with these conditions is still unclear, although an antibody driven or direct GABHS infectious hypothesis cannot be ruled out. Neuronal specific enolase is both cytosolic and membranous so an effect on neuronal membrane and particularly a functional effect of anti-neuronal antibodies cannot be ruled out. Further work is required, especially defining the cell type these antibodies bind to. It is probable that antibodies in Sydenham's chorea, PANDAS and TS are just a marker of disease mediated by streptococcus or indeed are just non-specific. Sydenham's chorea and TS may have different causes than autoimmunity.

The concentration of the antibodies is low compared with ant-neuronal antibodies associated with paraneoplastic disease. However, it is a similar titre to other autoantibodies such as ds DNA, ANCA, mitochondrial antibodies and others. Whilst antibodies are of low titre this does not imply that antibodies have no functional effect as complex interactions, including affinity of the antibody response are obviously important. Titration of SC samples against recombinant NSE is the next logical research step, although has considerable financial implications.

9 Discussion

9.1 Background

The primary aim of this thesis was to investigate the presence of anti-basal ganglia antibodies (ABGA) in Sydenham's chorea (SC) and the newly defined paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS). The similarity of PANDAS to Tourette's syndrome (TS) meant that the presence of ABGA could lead to the assessment of immunomodulatory treatments for this common disorder.

9.2 Evidence that Sydenham's chorea is an autoimmune disease

Studies by Witebsky *et al* in experimental thyroiditis led to the theory that autoimmunity could be defined by a number of scientific criteria (Witebsky *et al.*, 1957). These were: (1) the identification of autoantibodies or autoreactive cells; (2) The identification of a specific autoantigen; (3) The induction (with the use of immune adjuvants) of a similar disease in an experimental animal model (Witebsky *et al.*, 1957), (Table 9-1). These criteria were revised to include the passive transfer of autoimmunity into an animal model using serum or cells from a host with autoimmunity (Rose *et al.*, 1993), (Table 9-1). If SC is an autoimmune disease, then an equivalent set of criteria need to be fulfilled (Table 9-1).

Table 9-1 Criteria for defining autoimmunity and the evidence to support Sydenham's chorea and related conditions being autoimmune

Disease	Rheumatic fever	Sydenham's chorea	PANDAS	Tourette's syndrome
Presence of autoantibody	Yes	Yes	?	?
Identification of autoantigen	Yes Myosin and others	?Enolase and others	?Enolase and others	?Enolase and others
Induction of disease into animals	Yes	Yes (?)	?Conflicting data	?Conflicting data
Passive transfer of disease	Yes/No	?	Yes	Yes

The evidence for RHF being an autoimmune disease has not always been consistent but evidence now exists to fulfil each criterion (Table 9-1). The evidence for SC being an autoimmune disease has previously relied upon the presence of ant-neuronal antibodies in a small percentage of patients (Husby *et* al., 1976) and on incidental findings, such as the response of disease to immunomodulating agents such as corticosteroids. The identification of an autoantibody in a significant proportion of SC patients is an important step towards showing that SC and PANDAS and TS are also autoimmune. As TS and PANDAS appear to be phenotypically similar, the presence of an associated autoantibody may define an autoimmune subgroup, which could have major implications for

treatments of these conditions. In addition the identification of an autoantigen would be important for diagnosis and allow the creation of an animal model.

Concerning the important question as to why some patients develop chorea whilst others have PANDAS or TS, it was found that there was no difference in the mean age of onset between PANDAS, SC and TS patients. Age does not therefore appear to be a major factor. There was, however, a male preponderance in patients with tics (TS and PANDAS), whilst those with chorea were more likely to be female, as had been reported previously (Nausieda *et al.*, 1980). The difference in gender may mean that hormonal or subtle metabolic variations are important in the movement disorder phenotype, which manifests after a basal ganglia insult. There was no difference in the type of psychiatric manifestations between PANDAS and SC but the incidence of any psychiatric feature was higher in PANDAS. The main difference between SC and PANDAS was the presence of associated systemic disease in SC (RHF with carditis).

9.2.1 Additional evidence for autoimmunity in Sydenham's chorea

The data in this thesis does not rule out other immune mediators such as cells or indeed another mechanism being associated with chorea. Typically patients with SC do not have a CSF lymphocytosis (Prof. F.Cardoso, personal communication). CSF analysis of the UK samples did not reveal increased numbers of mononuclear cells, although a low grade lymphocytic infiltrate in the striatum may not necessarily be associated with a CSF pleocytosis. It is possible that cytokines, complement and other inflammatory mediators are an important feature of the pathogenesis instead of antibody binding *in vitro*.

In common with other CNS autoimmune disorders such as MS, CSF IgG-specific OCB were present in acute SC although, unlike MS, OCB were only found in 50% of the acute SC samples and there was no predominant pattern of results. This may reflect different antibody responses in individual cases of SC, blood-brain barrier function or the focal nature of the pathogenesis of SC. The SC patients with OCB all had higher cytokine levels than the other patients with SC, although the numbers tested were too small to draw definite conclusions. The sampling of CSF occurred at different time points in the disease course and this may also have influenced the results, as initial intrathecal synthesis of OCB may be followed later by a "mirror" pattern of OCB in CSF and serum or vice versa. One patient with acute SC underwent lumbar puncture 1 week after initial presentation, to rule out metabolic disease. Serum ABGA was negative at this time but CSF was positive for a 40 kDa band. A subsequent follow-up serum sample one month later was ABGA positive with a 40 kDa band (unpublished data).

None of the persistent SC patients had CSF or serum OCB, although the numbers tested were too small (four) to rule out the presence of OCB in a proportion of this group. Absence of OCB together with a normal serum cytokine profile in persistent SC supports my hypothesis that a significant number of cases of persistent SC do not show evidence of an immune pathogenesis and the chorea may instead be a reflection of neuronal damage from the initial insult or an alternative cause. The OCB assay is also designed to detect responses in disseminated demyelinating diseases such as MS and encephalitis where inflammation is more widespread (Walker *et al.*, 1983). As SC is a disease which

appears to affect only a focal region of the brain the detection limit of this assay may also be too low. It would be important to measure the IgG and albumin index in paired CSF and serum samples in addition to OCB. This would give some indication as to whether there is any blood-brain barrier damage allowing cytokines, antibodies and cells into the brain. I was not able to examine this due to limitations in sample volume.

In addition to historical reports, T lymphocytes, and a preponderance of B-cells have been reported in the brain of 1 case of ABGA associated with Parkinsonism and encephalitis following streptococcal infection (not invading the CNS), (Dale *et al.*, 2004). This may support the presence of an antibody-producing cellular response in some cases of post-streptococcal autoimmune movement disorders (Dale *et al.*, 2004). The presence of an inflammatory cellular infiltrate in post-streptococcal CNS syndromes may, however, represent severe and permanent pathologies rather than typical SC or PANDAS. Standard diagnostic imaging studies are normally unremarkable, whereas in paraneoplastic syndromes (probably cell mediated) there is usually evidence of brain abnormalities. Patients with evidence of a post-streptococcal movement disorder with an encephalitic onset (ADEM and focal encephalitis) appeared to be less likely to make full recoveries than those with typical SC and PANDAS (Dale *et al.*, 2001; Dale *et al.*, 2004). As ADEM has also been associated with a cellular response it may imply that a cell mediated neuronal loss is an uncommon finding in SC, PANDAS and TS.

Other surrogate evidence for a Th1 or Th2 immune basis for the pathogenesis of SC was investigated by investigating the cytokine levels. However, only a modest increase of any cytokine was present in acute SC with only 31% of patients having elevated Th1 or Th2 serum cytokines compared to controls. This low number could reflect the time of disease onset to sampling, the latency of the chorea post-infection and the assay sensitivity. Alternatively it may suggest that immune activation is not important in SC, or that the immune response is a focal rather than systemic, making evidence of immune activation difficult to detect. The CSF findings did, however, suggest a Th2 rather than a Th1 response in acute SC, as both IL-4 and IL-10 (Th2 cytokines) were raised. The important Th-1 cytokine, INF-γ, was undetectable in the acute SC group but this is difficult to measure accurately in body fluids so an undetectable level does not rule out a Th1 (cellular) response being important in the pathogenesis of SC.

Additional evidence for the importance of Th-2 cytokines and hence antibody production came from the persistent SC results, as 50% of these cases had raised CSF IL-4 levels, whereas other serum and CSF cytokines were within normal ranges. I also demonstrated that only approximately 50% of persistent cases are positive for ABGA. Therefore the only consistent finding in both acute and persistent SC was raised CSF IL-4. As IL-4 is a pivotal mediator of Th-2 responses, this may suggest that SC is more likely to be an antibody-mediated disease. Whether IL-4 is from peripheral sources and acts on B-lymphocytes sequestered in the brain, or is produced intrathecally in response to ongoing brain antigen presentation is not clear from this study.

In conclusion, a possible mechanism for disease induction (streptococcal autoimmunity) is that following an infection or persistent colonisation with GABHS, there is a local immune response to streptococcal proteins. In some patients, perhaps due to genetic susceptibility or immune hyper-responsiveness, this immune activation produces a number of antibodies and T cells which also recognise self-antigens. These inflammatory and autoimmune mediators can cause a host of immune-mediated syndromes including PSGN, vasculitides, arthritis, RHF and chorea, although the predominant immune mechanism probably varies. If the immune response is coupled with blood-brain barrier damage or increased barrier permeability with over-expression of adhesion molecules, inflammatory mediators can enter the brain. This may be influenced by genetic susceptibility or toxins from GABHS. Recognition of streptococcal epitopes with homology to human protein may cause ABGA to be produced as a result of molecular mimicry. As a result these B cells are activated within the CNS and B-cell specific cytokines can be detected in the CSF. Identification of the antigens against which ABGA are directed will lead to further studies to see whether or not this antibody response is directly pathogenic.

9.2.2 Anti-basal ganglia antibody studies in Sydenham's chorea using indirect immunofluorescence

The relatively low ABGA positivity rates in SC (Husby *et al.*, 1976; Kiessling *et al.*, 1993) are only weak support for an autoimmune criterion. This has cast doubt as to whether ABGA are truly associated with autoimmunity, or are simply an epiphenomenon secondary to basal ganglia damage. The ABGA response could just be part of a repertoire

of naturally-occurring antibodies found in GABHS infection or a response to local tissue inflammation or injury. In contrast to these earlier findings, a later study reported ABGA in 100% of acute and 93% of chronic SC using the same IF method (Kotby *et al.*, 1993). The discrepancy might relate to the subjective nature of the IF method used in these studies.

I found that ABGA are not basal ganglia specific but staining may be enriched in humans in this area, the titre of antibodies is low and typically 1/50 is an ideal screening titre. Using the IF method I developed with human basal ganglia, I found that ABGA were positive in 95-100% of patients with acute SC. This could be in accordance with a specific autoimmune syndrome but, is far too low a titre when compared to other anti-neuronal antibodies. Even though antibody titre was low, it was discriminating between SC, PANDAS, a subgroup of TS and controls so it might be a marker of disease in a similar fashion to anti-nuclear antibodies in connective tissue diseases such as SLE.

However, the IF studies conducted were just a pilot study due to lack of available funding. Therefore immunohistochemistry, confocal laser microscopy and electron microscopy using animal brain and/or striatal cultures could all be additional techniques to investigate. To determine if the neuronal-like staining present is afferent or efferent nerves, *in situ* hybridisation would be a useful technique to investigate as would multiple staining of sections with commercial antibodies against gabergic and dopaminergic markers.

To ascertain whether the presence of ABGA is related to possible genetic differences, I compared results from Brazilian patients with SC and those of a UK cohort of SC. The prevalence of ABGA did not differ between these cohorts. I did not detect the presence of other significant (systemic) autoantibodies in SC patients, suggesting that the ABGA response is at least brain-tissue specific.

It had not been previously shown that ABGA bound to neurones alone, as differentiation of the ABGA IF staining from glial (accessory) cells had not been performed in any published study. The localisation and identification of glial cells in tissue sections of human striatum was investigated by staining with an antibody specific for a glial cell marker (GFAP) and a neuronal antibody, neurone-specific enolase and comparing the results to ABGA staining. The results with GFAP or ABGA antibody showed that each antibody recognised different cell populations thereby suggesting that ABGA didn't appear to preferentially bind to astrocytes. The neuronal specificity of ABGA was shown by comparing the staining seen with ABGA with that of an antibody against NSE. The same pattern of staining was seen for both, showing that ABGA was neurone-specific. The similarity of patterns also suggested that a possible target autoantigen for ABGA might by NSE. Other antibodies need to be investigated to show this further, this wasn't done due to lack of money.

The weak staining in rat and monkey brain, especially cerebellar granule cells showed that ABGA are not striatum-specific. However, the striatum may just be an abundant source of the antigen compared to other areas of the brain. The staining I recorded in

human striatum might be more pronounced than in other areas, although this needs to be investigated systematically as was not done here. Another possibility is that certain neuronal types have a different expression of the target antigen i.e. cytoplasmic versus membranous distribution.

None of the normal controls was ABGA positive, although 10% of patients with rheumatic fever (without neurological symptoms) were positive with the same pattern that was seen in SC. Rheumtic fever and SC are inextricably associated, and another link with the clinical presentation of RHF and SC is that a high percentage of psychiatric symptoms have been found in RHF patients without evidence of SC (Mercadante *et al.*, 2000). It is tempting to speculate that there may be a relationship between ABGA positive RHF patients and those with psychiatric symptoms similar to that which occurs in SC (Freeman *et al.*, 1965; Moore., 1996; Swedo *et al.*, 1993). As no formal psychiatric assessment was made in the RHF patients I studied, this remains speculative but warrants further study, especially to ascertain why the RHF patients with ABGA can have psychiatric manifestations rather than obvious chorea. This might also be important in understanding the clinical and pathological relationship between SC and PANDAS.

There was no evidence of ABGA positivity in the neurological disease controls, which included: genetic, metabolic and vascular pathologies of the basal ganglia. This suggests that ABGA are not necessarily secondary to basal ganglia damage caused by a different pathological mechanism. However the numbers of controls with positive ABGA were higher in those with post-streptococcal, non-neurological, immune-mediated disorders.

The presence of ABGA could therefore be a risk factor for the development of any post-streptococcal autoimmune manifestation such as chorea, tics or psychiatric manifestations. Alternatively and possibly true is the notion that the anti-neuronal antibodies present in Sydenham's chorea might be a transitory response to a GABHS infection with too low a titre to have any functional role.

Indeed, other autoantibodies have been associated with immune-mediated syndromes other than that originally described. This means the presence of ABGA may not always indicate a neurological syndrome reducing its usefulness. For example, it has been shown that anti-myosin antibodies are present in both RHF and Kawasaki's syndrome, both of which are triggered by streptococcal infection (Cunningham *et al.*, 1999). The neurological symptoms related to ABGA may require the presence of antibody with additional triggers, such as damage to the blood-brain barrier allowing such inflammatory mediators access to the brain. Or an alternative cause for SC might be the direct effect of GABHS rather than any immune-mediated cause.

9.2.3 Anti-basal ganglia antibody studies in Sydenham's chorea using ELISA

The ELISA was developed as a potential screening assay for ABGA. The disadvantages of the ELISA were that it was not clear whether patients with streptococcal-associated CNS disorders had the same reactive antibody repertoire. Unfortunately false positive results could be caused by several factors, including polyclonal increases in serum immunoglobulins, antibodies which reacted with blocking reagents (heterophile antibodies) and the presence of systemic (non-brain specific) autoantibodies. To help

define whether the ABGA ELISA antibody response was specific, I also measured the IgG levels in SC patient serum, and found that they were not significantly different to controls. This was important as normal IgG levels would suggest that false positive, polyclonal binding to the basal ganglia antigens in the ELISA was producing the higher ELISA absorbances in SC. However, IgG was not normalised to age ranges and this is a criticism of this approach, but insufficient numbers in each age range were present to make meaningful comparisons.

An antigen preparation for the ELISA was optimised so that the antibodies detected were specific to basal ganglia proteins rather than lipids. This was because the lipid fraction of the basal ganglia homogenate contained elements of the myelin sheath. Antibodies against components such as myelin basic protein and myelin oligodendrocyte glycoprotein are a feature of a number of inflammatory CNS diseases, particularly MS and encephalitis (Berger *et al.*, 2003; Desai *et al.*, 1994; Egg *et al.*, 2001). These anti-myelin antibodies have also been reported in other inflammatory neurological diseases and are probably a consequence of neuronal damage (Reindl *et al.*, 1999).
De-lipidated, soluble fraction of whole basal ganglia homogenate was used as the ELISA antigen. This did discriminate SC patients from controls although some neurological disease controls also had raised ELISA result. This suggests that the ELISA was detecting antibodies against a number of different epitopes. The best discrimination between SC patients and control groups occurred when using de-lipidated basal ganglia, protein (soluble) enriched fraction. I believe this finding supports soluble protein(s) being the likely candidate antigen(s) in the ABGA binding seen on IF in SC patients. This was

confirmed by testing the insoluble basal ganglia homogenate fraction which did not discriminate between SC and controls.

During the development of the ABGA ELISA I also used a negative cut-off value (upper limit of normal) to assess the proportion of samples with raised ELISA results above the upper-limit of normal. This was important as the differences in mean ABGA absorbance may only reflect high absorbances in a small percentage of patients rather than in the majority of the cohort. The cut-off allowed semi-quantitative analysis of the ABGA results. The ELISA appeared to be as sensitive as IF when detecting ABGA in SC but specificity was not sufficiently high for the assay to be reliable.

The healthy and neurological control groups were both negative on ABGA ELISA using the cut-off value. However, 21% of streptococcal-associated autoimmune diseases control groups had raised ELISA results which mirrored IF findings. The RHF group also had similar results to those seen when using IF, as up to 20% of patients were positive compared to the ELISA cut-off. The number of UK controls with streptococcal-associated autoimmune conditions (PSGN, arthritis and vasculitis) who were ABGA ELISA positive was small, but higher than that seen from normal or mixed neurological disease controls. The specificity of the ABGA ELISA appears to be acceptable in normal and neurological control groups, with a typical specificity of approximately 90%. This was not mirrored in streptococcal controls, so the ELISA is not a suitable screening test and is not ideal for identifying ABGA.

9.2.4 Anti-basal ganglia antibodies in Sydenham's chorea using Western immunoblotting

IF is a subjective method and ELISA was unable to distinguish between antibody bound to single or multiple epitopes. A Western immunoblotting method was therefore developed using the same human basal ganglia antigen as ELISA in order to detect any specific ABGA responses to the same protein. The soluble S1 fraction was used. The most frequent basal ganglia antigens recognised by serum from both acute and persistent SC patients had molecular weights of 40, 45, 60 and 98 kDa. The healthy adult controls and children with developmental delay did not have any specific reactivity against basal ganglia Western immunoblots. By comparison, approximately 30% of RHF patients had specific reactivity against basal ganglia proteins, and appeared to have the same bands that were present in SC. This confirms that some patients with RHF, without obvious CNS symptoms, have ABGA. This again suggests that IgG responses against brain might be related to GABHS infection, but doesn't suggest a functional role as candidate antigens are soluble and from the cytoplasm.

9.3 Comparison of these ABGA results with current research

Following publication of the ABGA study in SC (Church *et al.*, 2002; Church *et al.*, 2003) another group also reported an ELISA and Western immunoblotting method for detecting ABGA (Singer *et al.*, 2003). This group reported higher ELISA absorbances in their SC patients but these were not statistically different from controls (Singer *et al.*, 2003). They also used a cut-off value using the same method as in this thesis (Church *et al.*, 2002; Singer *et al.*, 2003) and reported that only 33% of SC patients had raised

ELISA absorbances against caudate and putamen antigens. Using Western immunoblotting they found no specific protein band exclusive to SC or controls and measuring the total area under each band using densitometry also failed to show significant differences (Singer *et al.*, 2003). However, a statistical, multi-variant analysis of their results did show discrimination (ABGA positivity) between SC and controls. The use of soluble homogenate from the caudate had the greatest power of discrimination (Singer *et al.*, 2003). My IF studies found that antibody (IgG) from SC patients predominantly bound to the caudate. The molecular weight of proteins which gave the highest statistical discrimination in the study by Singer *et al* was reported to be 126 kDa and 113 kDa. I had not identified these proteins in any Brazilian or UK patients.

The difference between Singers *et al's* results and mine is the lower proportion of SC patients who were ABGA positive in the former. This could be reflected in the preparation of tissue for ABGA antigens. Singer *et al* performed their ABGA ELISA against separate fractions of caudate, putamen and globus pallidus whilst mine contained whole caudate and putamen. My antigen source may therefore have contained more of the target antigen because an intact striatum rather than a dissected one was used. There were also fundamental variations in methodology when preparing the homogenates for Western immunoblotting. I found that including a commercial detergent cocktail (T-per Pierce) improved the recovery of proteins compared to simple mechanical homogenisation. Singer *et al* used 10% homogenous PAGE gels for their Western immunoblotting whilst I found that a gradient PAGE gel (4-12%) increased the separation of proteins which gives better resolution. Discrimination between 2 closely

aligned bands was easier in a gradient gel. I also found that IgG from the donor tissue reacted with the secondary detector antibody (anti-human IgG). As this was an expected result this band was always present and hence excluded from the analysis of differences between SC patients and controls in my results. This may have caused problems in the analysis of results in Singer *et al's* Western immunoblotting study.

Another reason for possible differences in results between the two studies may relate to the lipid fraction of the basal ganglia homogenate. Unlike Singer *et al*, I removed the lipid fraction before using the remainder for the detection of ABGA. The problems with identifying a specific signal were also shown from the ELISA results using the lipid fraction which was not discriminating. This may explain the multiple bands in SC patients and controls reported using crude basal ganglia homogenates (Singer *et al.*, 2003). The methods for detecting antibodies may also influence the results from different studies. Singer *et al* used enhanced chemiluminescence to detect ABGA in Western immunoblotting, which is a very sensitive method but can produce a multitude of responses in patients and controls, most of which are of doubtful significance. I proposed that colorimetric analysis of Western blots in this setting is preferable and improves specificity, although arguably at a cost of reduced sensitivity.

An alternative explanation for the presence of ABGA in SC, PANDAS and TS is that it occurs as an epiphenomenon secondary to basal ganglia damage. I believe this is unlikely as the control groups that were tested had inflammatory, metabolic and ischaemic basal ganglia diseases and were ABGA negative. In addition to the ABGA and streptococcal

data in TS, a provocative study was carried out by Hallett and co-workers, in which they microinfused the sera or IgG from patients with TS into the striatum of rats to ascertain it effects on movement. This induced stereotypies which were proposed to be analogous to the involuntary movements seen in TS (Hallett *et al.*, 2000). This was confirmed in a separate study of TS sera (Taylor *et al.*, 2002). However, a recent study could not induce behavioral changes in a rat model (Loiselle *et al.*, 2004). As I have found that the ABGA repertoire (40, 45, 60 and 98 kDa proteins) is similar in post-streptococcal tics or choreiform disorders, these observations suggest that ABGA may play a pathogenic role in SC, PANDAS and a subgroup of TS. This is important as it may be a useful laboratory test in patients presenting with idiopathic movement disorders.

9.4 Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections

All the PANDAS patients studied had a IF binding pattern against basal ganglia neurones similar to that previously reported in SC and seen in my studies (Church *et al.*, 2002; Church *et al.*, 2003; Husby *et al.*, 1976; Kotby *et al.*, 1998). This may suggest that regardless of the phenotype of the movement disorder, (SC or PANDAS), the associated ABGA response is essentially the same. It is not surprising that ABGA could produce a range of extra-pyramidal movements and psychiatric manifestations, as rarely do diseases affecting the basal ganglia result in just a single movement disorder phenotype (van Dijk *et al.*, 1986).

The mean ABGA ELISA result in the PANDAS group was elevated in every comparison to the results from the neurological, streptococcal, autoimmune and normal controls. In addition, analysis of the PANDAS group showed that the mean ABGA ELISA derived a sensitivity of 82.4% and specificity of 79%. Analysis of the Western blotting results indicated that the same antigens (recognised by molecular weight) were present in PANDAS as SC. Interestingly the 40 kDa basal ganglia antigen was more common in patients with PANDAS than SC, so this may yet prove to be a useful marker for PANDAS.

ABGA was prevalent during the active phase of PANDAS and decreased during remissions (where follow up samples were available). This phenomenon may be important when attempting to identify autoimmune subgroups in other chronic neuropsychiatric syndromes where post-streptococcal autoimmunity is implicated, such as TS or OCD. This is important in chronic disorders as ABGA may only be present early on in the disease when it could cause permanent damage to neurones. The ABGA ELISA method was not useful in these chronic settings as sensitivity of the assay was too low. Western immunoblotting can define specific antigenic binding and I found this method, in conjunction with IF, to be the preferred marker.

In conclusion the results of this study suggest that ABGA Western immunoblotting and IF can be used as a marker in investigating patients with movement disorders where streptococcal infection has either been implicated as an initial trigger or associated with exacerbation of symptoms. This implies that cases of idiopathic dyskinesias where other

causes have been eliminated should be investigated to see whether they have ABGA and Streptococcal infection. Importantly the range of hyperkinetic movement disorder should not be limited chorea or tics, as patients with other movement disorders, particularly dystonia have ABGA and evidence of recent streptococcal infection (Dale., 2003; Church *et al.*, 2002).

9.5 Tourette's syndrome

It has been reported that patients with TS also have significantly higher rate of positive streptococcal serology results (ASOT and DNAse B) compared to control subjects (Cardona *et al.*, 2001; Morshed *et al.*, 2001; Muller *et al.*, 2000) thus supporting a hypothesis that TS could also be a post-streptococcal immune-mediated disorder. It is important to note that, as with SC, not all TS studies have reported a consistent elevation in streptococcal serology (Singer *et al.*, 1998; Singer *et al.*, 1999). To investigate the incidence of streptococcal infection one group investigated antibody reactivity to streptococcal M-proteins in TS (Muller *et al.*, 2001). They demonstrated elevated antibody titres to M12 and M19 proteins using an ELISA, but not to M1, M4 and M6 in 25 adult TS patients compared to 25 control subjects (Muller *et al.*, 2001). This lends further support to the concept that particular strains of GABHS are associated with these disorders. Strain specificity is compatible with the observations that other post-streptococcal immune-mediated disorders, i.e. RHF and PSGN are also strain-specific (Stollerman., 2001).

Increased antibody binding to putamen rather than caudate had been reported in TS, and basal ganglia antigens of molecular weights 60, 67 and 83 kDa have been suggested as common epitopes (Singer *et al.*, 1998). A further study also suggested that 60 kDa was the most prevalent basal ganglia autoantigen in TS although a complex multivariate analysis was required to establish an association (Wendlandt *et al.*, 2001). Preliminary results from another group also suggested that responses to 60 kDa and 83 kDa antigens were dominant in patients with tics, TS and/or OCD (Trifiletti *et al.*, 2000). The same authors suggested that these antibodies recognized a calpain-calpastatin complex (Trifiletti *et al.*, 2000 abstract). The consistent finding of a 60kDa antigen from my SC and PANDAS studies suggests that the 60 kDa antigen could be important in SC, PANDAS and TS.

In view of the conflicting ABGA results in TS, a large cohort of TS patients and various control groups was investigated using for the presence of ABGA. Results showed that 27% of adult TS, 20% of children with TS and 2-4% of control groups were ABGA positive by Western immunoblotting. This was confirmed by IF of the positive samples. The IF staining pattern was the same as that seen in SC and PANDAS, with staining predominantly confined to the caudate rather than the putamen. The low percentage of ABGA positivity in TS compared to SC and PANDAS supports the heterogeneous nature of TS pathogenesis. As only approximately 20% of patients in this study were ABGA positive this could explain why many studies have found different results for ABGA and streptococcal serology (b. Church *et al.*, 2003). A large cohort of TS patients needs to be

studied to provide consistent results or to identify the small proportion of TS patients who have evidence of streptococcal infection and ABGA.

My results do not support the putamen as the predominant ABGA binding site in TS, unlike those of Singer *et al.* The putamen results were obtained from ELISA and Western immunoblotting methods so the differences could be related to antigen preparation. Contamination of putamen antigens by caudate proteins, perhaps during post-mortem removal of the striatum, may explain why they also reported a 60 kDa antigen common to TS patients (Singer *et al.*, 1998). Using ABGA ELISA I found no difference in the mean absorbances between children and adults with TS compared to controls, and elevated ABGA was found in 16% of children and 11% of adults. This was not significantly different from controls. The failure of ELISA to discriminate between TS and controls suggested that either ABGA or streptococcus (or both) are not important in the pathogenesis of TS or they are important in only a small subgroup of patients. Western immunoblotting and IF, however, did discriminate and identified a proportion of patients with TS who were ABGA positive. The failure of ELISA to do this may be related to sensitivity of the assay, or TS may simply be associated with low concentrations of ABGA. Another possibility relates to the nature of the study, which was cross-sectional and recruited patients who had had TS for many years. That the fact that ABGA positivity was higher in children suggested that ABGA might be lost with time which is similar to the observations in persistent SC. ABGA may therefore only be important in the initial disease induction and chronic TS may simply be a consequence of damage to

the basal ganglia. Alternatively ABGA might just be related to GABHS infections and have no role at all.

9.6 The importance of streptococcal infection

The production of ABGA may be influenced by the streptococcal strain causing the infection, and unidentified co-factors and host susceptibility to autoimmunity. Genetic susceptibility probably plays a role in view of the familial association of SC and RHF (Read *et al.*, 1938; Wilson *et al.*, 1954) and the observation that specific racial groups appear to have increased susceptibility to RHF and SC (Carapetis *et al.*, 1999; Haidan *et al.*, 2000). No reproducible association with HLA has yet been found in SC or RHF (Donadi *et al.*, 2000). Similarly, the presence of the putative B-cell marker D8-17 has also not been confirmed in RHF, SC or PANDAS.

Supporting evidence for the involvement of streptococcal infection in the SC patients studied came from the clinical observation of active carditis, previous streptococcal pharyngitis and in one case, GABHS skin infection. The SC patients from Brazil did not undergo routine throat cultures to detect GABHS. In 3 patients from the UK SC group GABHS was isolated from the throat. As the numbers of SC patients with raised streptococcal serology varied between historical studies (Ayoub *et al.*, 1966; Eshel *et al.*, 1993; Moore., 1996), I measured both ASOT and anti-DNAse B to address this issue. No acute SC patient had both negative ASOT and anti-DNAse B compared to cut-off values. This demonstrated, albeit using serology, that evidence of GABHS was prevalent in the acute SC group and at a significantly higher rate than in normal subjects and neurological

disease controls. The fact that ASOT or anti-DNAse B positive serology were relatively the same in acute SC patients could not be used to distinguish between past or present streptococcal infection and whether either is associated with SC. If SC is a manifestation of past infection one would expect the anti-DNAse B to be raised more frequently than ASOT. This is because ASOT declines soon after infection, whilst anti-DNAse B remains elevated for a longer period of time (up to 3 months).

Alternatively acute SC may be associated with persistent streptococcal infection or colonisation rather than a latent immune manifestation of a previous infection. This may have caused ASOT levels to remain elevated due to difficulty in clearing the infection. Evidence of the nature of GABHS infection in SC could be inferred by comparing to the rates in other GABHS pathologies. Serological evidence of streptococcal infection in other streptococcal diseases such as uncomplicated pharyngitis was similar to SC. This may indicate that acute infection or re-infection could be associated with some cases of acute SC. However, the rate of positive streptococcal serology in SC was similar to the rates in other autoimmune post-streptococcus disorders such as PSGN which are thought to be latent manifestations of streptococcal infection. This suggests that previous infection with a latent immune activation can also be the cause of some cases of SC. The streptococcal serology in persistent SC patients showed a lower percentage of raised ASOT and anti-DNAse B than in acute SC. This was the same for ABGA positivity. This supports the hypothesis that approximately half of the persistent SC cases are probably not driven by very recent streptococcal infection and/or the presence of ABGA.

Interestingly, persistent SC cases who were ABGA positive were more likely to have a raised ASOT and/or anti-DNAse B.

In conclusion the immune response to GABHS which results in SC may be related to a particular individual's immune response. Instead of SC being a latent immune manifestation, acute infection or persistent colonisation may be equally important but in different patients. Persistent colonisation may occur due to the ability of the streptococcal strains to internalise and colonise cells in the nasopharynx, so using ASOT or anti-DNAse B to interpret the time-course of infection may be unreliable. The variation in immune responses to GABHS might also be the reason why some patients with GABHS develop the ABGA response against the 40, 45 and 60 kDa proteins and some do not. Therefore ABGA might just be a response to infection, self limited and of no importance other than to be a marker of severe complications of infection such as RHF and SC.

Streptococcal serology results in patients with PANDAS was similar to that of patients with SC, i.e. a higher proportion of patients with PANDAS had raised ASOT and anti-DNAse B compared to patients with other neurological diseases or other control groups. Results from the normal control groups confirmed that streptococcal infection is common in childhood. Evidence from either serology or throat culture and a clinical history of pharyngitis indicate that GABHS infection is very common in patients with PANDAS. This supports the results of others (Swedo *et al.*, 1998).

Unexpectedly the majority of the subjects with TS had raised ASOT, although only a minority were ABGA positive, indicating that a higher proportion of patients with TS have evidence of recent or persistent streptococcal infections. If, as I suspect, ABGA are strongly related to streptococcal infection, the failure to find a higher proportion of patients who were ABGA positive may relate to the sensitivity of the ABGA assays. Alternatively, ABGA levels may fluctuate during the course of TS, possibly mimicking the waxing and waning of symptoms and signs of TS (Robertson., 2000). Indeed 6/7 (85%) of the TS patients I followed with raised initial ASOT, but who were negative for ABGA, seroconverted and became ABGA positive. This coincided with a reduction in the ASOT, 3 months after the initial sample. A longitudinal study involving large numbers of known TS patients is underway to address the temporal association between ABGA-streptococcal infection and clinical course.

Unfortunately the anti-DNAse B assay was not available for this study. Serological evidence of recent GABHS infection was detected in 91% of patients with positive ABGA compared to 57% of patients who were negative for ABGA. The patients did not have routine throat culture as part of this study. If ABGA and a positive ASOT in patients with TS are related it is uncertain at present why ABGA are not found in all TS patients with evidence of recent streptococcal infection. A simple explanation may be that the current ABGA assay has a low sensitivity or that these responses are just transitory and of no importance. This will need to be explored using ELISA with a recombinant human protein as the antigen source. A high carriage rate of GABHS in patients with TS is intriguing and is persuasive evidence to support the argument that GABHS plays a

pathological role in TS. The implications for this work are that alternative treatment strategies such as antibiotics or immune-modulating drugs such as steroids or IVIG may be useful in managing and alleviating the symptoms of TS.

A plausible alternative to the hypothesis that proposes ABGA as being the cause of these syndromes is that the presence of a streptococcal toxin such as streptolysin O, or a streptococcal pyrogenic exotoxin, might be responsible for the brain pathogenesis. There is evidence that a toxin plays a role in other streptococcal immune-mediated syndromes such as scarlet fever and Kawasaki's syndrome. In these syndromes streptococcal toxins are implicated directly in cell death and indirectly via activation of the immune response and innocent-bystander damage of cells. Streptolysin O, for example, binds to cholesterol on cell membranes resulting in toxin-cholesterol aggregates which cause cell lysis through an osmotic mechanism (Bhakdi *et al.*, 1985; Palmer *et al.*, 1998; Sekiya *et al.*, 1993; Sekiya *et al.*, 1996). Significant concentrations of streptolysin O in the brain could cause cell death or through binding to cholesterol cause a change in cell potential and disruption of neuronal function. As there is no evidence to support brain colonisation of GABHS, extracellular toxins would have to enter the brain via the vasculature.

A novel streptococcal toxin might be binding to a specific ligand only found on basal ganglia neurones, perhaps causing dysfunction of the dopaminergic pathway thus leading to complex movement disorders. A specific strain of streptococcus may therefore be capable of producing a neuronal toxin, and this could explain the elevated streptococcal serology. To date no novel GABHS strains have been found in these patients but due to

the constantly changing pattern of virulence in streptococcus species, this might be less important than the ability to produce a particular toxin.

The presence of antibodies (ABGA) might also be related to an immune response to a toxin that binds to neurones. A similar pathogenesis had been proposed in post-streptococcal nephritis (Rincon *et al.*, 2003; Treser *et al.*, 1969). To test this hypothesis the effect of extracellular toxins on neuronal cultures could be investigated. The particular streptococcal strains isolated from patients with SC, PANDAS and TS could also be investigated to see if they contain novel genes which differ from strains associated with uncomplicated infection.

9.7 Putative autoantigen

The identification of the candidate proteins that ABGA bind may give some insight into the pathological relationship between the streptococcus and the brain. The cellular distribution of the proteins that ABGA recognised may also be important as a cytoplasmic localisation would cast more doubt on the pathological significance of these antibodies. It is unclear at present whether autoantibodies directed at intracellular antigens can affect the cell function. The identification of the proteins I found most commonly associated with ABGA binding in SC, PANDAS and TS was thus the next important step.

The isolation and identification of the candidate antigens (recognised by ABGA using human tissue) was problematic due to difficulties in obtaining large quantities of human

tissue. Animal tissue would have been a usual route to identify putative antigens but as IF staining was weak against rat tissue, human was used. Sera which appeared to react against a single basal ganglia protein: 40, 45, 60 or 98 kDa were tested separately to facilitate identification of reactivity. The 45 kDa protein was found at the acidic end of IEF gel (pI 4-5) and reactivity was co-associated with the 98 kDa protein, even though not all sera reactive with the 98 kDa band had evident reactivity against a 45 kDa band using standard Western immunoblotting. Using 2-D electrophoresis probably increased the quality and quantity of protein separation, making the positivity of samples to both the 45 and 98kDa bands more apparent. The 2-D electrophoresis pattern showed that there were 2 reproducible protein 'spots' which corresponded to 45 and 98 kDa molecular weights. I did not find these proteins were reactive when testing control serum samples. The mass-spectroscopy analysis of the 2 spots identified them as the monomer and dimer of the same protein, neurone-specific enolase (NSE).

In comparison I used the same methods to investigate the 40 and 60 kDa bands. These were difficult to isolate from the 2-D gels as they migrated to a point where there were multiple, sometimes overlapping proteins. I decided to use different methods of protein separation and isolation by using immunoprecipitation. Unfortunately, despite using the maximum concentrations of antigen and antibody I was unable to extract target proteins in sufficient quantities to identify by mass-spectroscopy. I tried to overcome these problems by concentrating the proteins obtained from the immunoprecipitation method but the amino acid sequence still could not be determined because of the low protein concentration. I was unable to concentrate the proteins further as the mass-spectroscopy

system required a minimum sample volume. These results may imply that despite clear immunoreactivity using my methods, the 40 and 60 kDa proteins are not useful discriminate protein targets in streptococcal CNS disorders. Alternatively the concentrations of the antigen in neurones may be low. However Dale *et al.*, using immunoprecipitation and Fluid Phase Liquid Chromatography of rat brain, identified these proteins as Pyruvate kinase M1 and Aldolase C (Dale *et al.*, 2006). There may of course be more important antigens than those described but different methods are required to examine this hypothesis further.

An alternative method for identifying autoantigens would be to create hybridomas from peripheral lymphocytes and produce monoclonal antibodies which could be screened against streptococcal proteins. Kirvan *et al* using this technique have recently shown that a monoclonal antibody derived from one patient with SC recognised streptococcus GlcNAc (Kirvan *et al.*, 2003). This antibody recognised lysoganglioside. As only one patient was used to produce this monoclonal antibody it is not known if this is a common finding in SC or whether the patient had had a previous disorder, for example GBS, which could have been responsible for the anti-ganglioside antibodies. The ability of this patient's CSF to activate CAM II kinase (Kirvan *et al.*, 2003) may not be related to the monoclonal specificity of the ganglioside antibody but may be secondary to other immunoglobulin reactivity.

9.8 Neurone-specific enolase

The identification of NSE as a putative autoantigen is interesting as a homologue of enolase is found on the cell surface of streptococci. This could support molecular mimicry as a method of antibody induction but does not imply a functional role. A comparison of high power IF between a commercial anti-NSE antibody and ABGA showed similar staining patterns. Interestingly, cell surface enolase has been reported on both encapsulated and un-capsulated streptococci but not staphylococci (Pancholi *et al.*, 1993). This has been used to suggest that enolase is an important feature of respiratory pathogens (Pacholi *et al.*, 1998). The role of glycolytic enzymes such as enolase on the cell surface of bacteria is unknown at present, but enolase can bind plasminogen so it may play a role in bacterial attachment and infection (Derbise *et al.*, 2004; Fontan *et al.*, 2000).

Antibodies raised to streptococcal surface enolase have been shown to react with human α-enolase (Fontan *et al.*, 2000), which is found in the cytoplasm of all human cells (Kato *et al.*, 1983). It has also been reported that levels of antibodies which react with human α-enolase and streptococcal enolase are raised in patients with RHF compared to those with uncomplicated streptococcal pharyngitis or normal controls (Fontan *et al.*, 2000). To date no studies have investigated antibodies against gamma enolase (NSE) in post-streptococcal sequelae. Alpha and gamma enolase are isoforms, so why antibodies reacting to alpha enolase are found in RHF, whilst gamma enolase (neuron-specific) might be present in SC is unknown. It may be that antibodies recognise different enolase epitopes, so epitope mapping of both alpha and gamma enolase would be required to

answer this question. This may also be important in RHF and PSGN as there may be heart-and kidney-specific enolase isoforms. Indeed, antibodies against alpha-enolase from human kidney extracts have been found associated with kidney disease in mixed cryoglobuinemia (Sabbatini *et al.*, 1997). Alternatively, differences in the streptococcal enolase protein between rheumatogenic and neurogenic streptococcal strains might be responsible for the different antibody profiles in RHF and SC. Studying the GABHS serotypes in each syndrome would be required to investigate this hypothesis.

9.9 Speculation as to the role of enolase

It was surprising that the 45 and 98 kDa basal ganglia proteins I identified were NSE rather than a protein specific just to the basal ganglia. The results from the ABGA IF suggested that the antibody response was linked to a specific or enriched source of antigen in the basal ganglia. This may be because the relative concentration of NSE is higher in a particular type of neurone in the basal ganglia. However, this is probably unlikely, since a histochemical study showed that NSE (gamma enolase) was present in neurones throughout normal human adult brain (Joseph *et al.*, 1996; Royds *et al.*, 1982).

However, although NSE is a cytoplasmic protein, a membranous distribution in neurones has also been reported (Lim *et al.*, 1983, Leung *et al.*, 1987). An important finding related to post-streptococcal basal ganglia autoimmunity is that multiple monoclonal antibodies raised against NSE have shown a different distribution of NSE within the brain (Frikke *et al.*, 1987). It was suggested that there might be multiple conformational or structural forms of NSE, perhaps related to post-translational modification (Frikke *et*

al., 1987). Therefore NSE expressed in basal ganglia neurones may be a different form, or possess different epitopes on the cell surface, to that in other areas of the brain.

There have been no studies as to the membrane distribution, concentration or exact role of NSE in different areas of the brain. A membrane localisation of NSE could be more pronounced in the caudate or a particular type of neurone in the basal ganglia. The distribution and levels of expression of NSE may be linked to the metabolic requirements of the basal ganglia since NSE is a glycolytic enzyme capable of producing ATP actually on the membrane (Lim *et al.*, 1983). Indeed NSE exists as both monomers and dimers on the neuronal membrane surface (Ueta *et al.*, 2004). This has lead to a hypothesis that glycolytic enzymes might have multi-functional "moonlighting" roles (Pancholi *et al.*, 2001). The reason why striatal enolase might be a different isoform from typical NSE might be related to the growing notion that many proteins have "moonlighting effects", i.e. proteins can have functions different from that normally expected (Copley., 2003). Therefore striatum, NSE might have novel functions, perhaps mediated through a channel such as $N^{+}K^{+}$ATPase (Dale *et al.*, 2006).

Dale *et al* showed increased apoptosis in cerebellar granule cells (Dale *et al.*, 2006). They suggested that local energy failure, mediated by anti-enolase antibodies effecting ATP production on striatal, neuronal membranes could lead to cell death or other metabolic consequences. A functional effect of anti-NSE antibodies in subjects with SC might be interruption in glycolysis. However, anti-NSE antibody had no immediate, significant effect on enzyme. Provocatively, magnetic resonance spectroscopy studies have reported

increased glucose turnover and hypermetabolism in the basal ganglia of patients with SC (Goldman *et al.*, 1993; Lee *et al.*, 1999; Weindl *et al.*, 1993) which could support a subtle energy distruption. A case of ABGA associated with minor chorea and a dementia like presentation was assessed by positron emission tomography which also showed hypermetabolism in the left striatum with a normal MRI (Léger *et al.*, 2004). Treatment with corticosteroids improved her condition and the PET scan showed a return to normal in the left caudate (Léger *et al.*, 2004).

These studies could suggest a hypothesis of local metabolic stress in the striatum in SC due to antibody binding and interruption of metabolic pathways. This causes disruption in the neuronal control of normal movements, resulting in development of chorea and tics. A hypothetical pathway of antibody effect is shown in figure 9-1 and 9-2. Metabolic effects of antibody binding to enzymes (Figure 9-2) could be causing an effect on a membrane receptor as enolase can produce ATP on the cell surface (Lim *et al.*, 1983). A possible hypothesis is local energy failure resulting in alterations of neuronal membrane function or apoptosis of neurones with medium spiny striatal neurones susceptible to such changes (Dale *et al.*, 2006). As no direct effect on ATP production was shown in this thesis how energy failure might occur is unknown but it might be due to an accumulative effect of antibody binding.

Figure 9-1 Summary of possible mechanism for anti-neuronal antibody effects in Sydenham's chorea

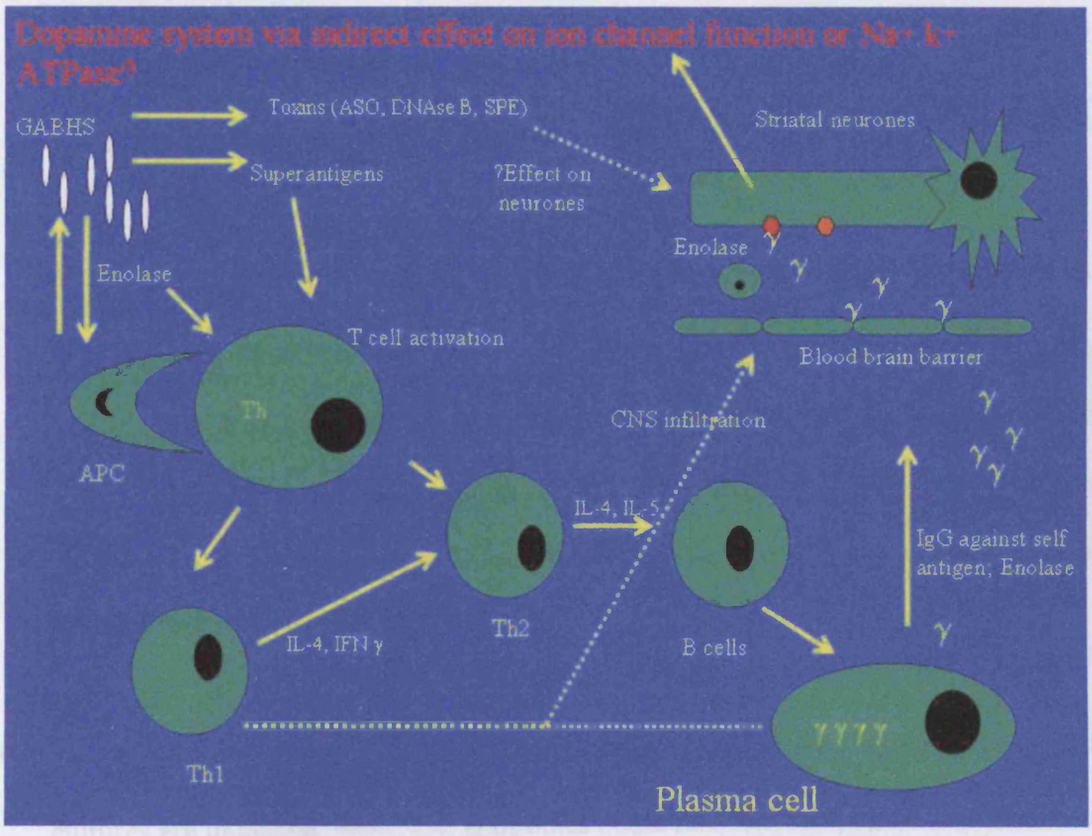


Figure 9-2 Role of enolase in glycolysis

- 3-Phosphoglycerate↔2-Phosphoglycerate (Phosphoglyceromutase)
- 2-Phosphoglycerate↔phosphoenolpyruvate+H_2O *production of high energy phosphate* **(enolase)**
- Phosphoenolpyruvate+ADP→pyruvate+ATP *production of pyruvate* **(pyruvate kinase)**

Whilst enolase and pyruvate kinase are enzymes that appear in stage II of glycolysis, aldolase C is a stage I enzyme

- D-Fructose 1,6-diphosphate↔dihydroxyacetone phosphate+D-glyceraldehyde 3-phosphate *adol condensation yielding 2different triose phosphates* **(Aldolase C)**

The glycolytic enzymes suggested as antigens in post-streptococcal neurological disorders are shown in their glycolytic role in bold type.

To examine a metabolic hypothesis further, *in vitro* experiments, using neuronal cell cultures are indicated. Anti-NSE antibodies could be introduced into a cell culture and, using a fluorescent antibody marker, the cellular distribution of the anti-NSE antibodies could be investigated over time. This would show whether or not ABGA bound primarily to the surface enolase before being endocytosed and localised in the cytoplasm. It would be useful to study the enolase homologue expressed by GABHS as it is not known whether all GABHS strains express enolase. Strain specificity in post-streptococcal CNS disorders may be related to the proteins expressed on the bacterial cell surface in conjunction with susceptibility to autoimmunity or hypersensitivity following infection. Further investigation of NSE as a possible target autoantigen would involve injecting the protein or peptides with adjuvant aimed at producing an animal model of CNS disease.

Injecting homogenates of GABHS have also been proposed in a murine model of PANDAS, eliciting an antibody response which bound to deep cerebellar nuclei (Hoffman *et al.*, 2004).

9.10 Final Conclusion

In conclusion to determine the incidence and prevalence of streptococcal-associated CNS disorders associated with anti-NSE antibodies might be interesting to pursue further. The ideal method would be to use recombinant NSE protein in an ELISA, as human NSE is difficult to obtain commercially, expensive and purification of large quantities of NSE from human material is impractical. Samples with suspected positive anti-NSE antibodies could be confirmed by Western immunoblotting analysis using the same recombinant NSE.

Anti-basal ganglia antibodies do not appear to be specific to basal ganglia. However, an IgG antibody directed at neuronal glycolytic enzymes has been found in SC and PANDAS. Unlike paraneoplastic syndromes this antibody is of weak concentration although the affinity of these antibodies is unknown. The presence of this antibody probably represents an acute phenonomen related to streptococcal infection and the incidental presence of a neurological, extrapyramidal movement and psychiatric disorder. Further work, including longitudinal studies, is required to answer the relationship between these antibodies and clinical phenotypes.

Other work which may be carried out is to look for anti-neuronal antibodies directed to a channel. If functional autoantibodies exist in SC they might be directed against a neuronal membrane channel, so RIA assays of calcium or potassium channel antibodies is required. If other immune-mediated causes for SC and PANDAS are considered, T-cell responses need to be investigated. Animal studies of enolase antigen are required if enolase is accepted as an antigen. Histopathology is required in great detail to look at antibody binding.

References

Adderson EE, Shikhman AR, Ward KE, Cunningham MW. Molecular analysis of polyreactive monoclonal antibodies from rheumatic carditis: human anti-N-acetylglucosamine/anti-myosin antibody V region genes. J Immunol 1998 Aug 15; 161(4): 2020-2031.

Afanasyeva M, Wang Y, Kaya Z, Park S, Zilliox MJ, Scholfield BH, Hill SL, Rose NR. Experimental autoimmune myocarditis in A/J mice is an Interleukin-4-dependant disease with a Th2 phenotype. Am J Pathol 2001 Jul; 159(1):193-203.

Akesson P, Sjoholm AG, Bjorck L. Protein SIC, a novel extracellular protein of Streptococcus pyogenes interfering with complement function. J Biol Chem 1996 Jan 12; 271 (2): 1081-1088.

Albert ML, Darnell JC, Bender A, Francisco LM, Bhardwaj N, Darnell RB. Tumour-specific killer cells in paraneoplastic cerebellar degeneration. Nat Med. 1998 Nov; 4 (11): 1321-1324.

Albin RL, Young AB, Penney JB. The functional anatomy of basal ganglia disorders. Trends Neurosci 1989 Oct; 12 (10): 366-375.

Alexander GE, DeLong MR, Strick PL. Parallel organization of functionally segregated circuits linking basal ganglia and cortex. Annu Rev Neurosci 1986; 9: 357-381.

Alouf JE, Loridan C. Production, purification, and assay of streptolysin S. Method Enzymol 1988; 165:59-54.

Antone SM, Adderson EE, Mertens NM, Cunningham MW. Molecular analysis of V gene sequences encoding cytotoxic anti-streptococcal/anti-myosin monoclonal antibody 36.2.2 that recognizes the heart cell surface protein laminin. J Immunol 1997 Dec 1;159 (11): 5422-5430.

Aron AM, Freeman JM, Carter S. The natural history of Sydenham's chorea: review of the literature and long term evaluation with emphasis on cardiac sequelae. Am. J. Med 1965 Jan; 38: 83-95.

Asbahr FR, Ramos RT, Negrao AB, Gentil V. Case series: increased vulnerability to obsessive-compulsive symptoms with repeated episodes of Sydenham chorea. J Am Acad Child Adolesc Psych 1999 Dec; 38 (12):1522-1525.

Ayoub EM, Wannamaker LW. Evaluation of the streptococcal desoxyribonuclease B and and diphosphoyridine nucleotidase antibody tests in acute rheumatic fever and acute glomerulnephritis. Pediatrics 1962; Apr: 527-538

Ayoub EM, Wannamaker LW. Streptococcal antibody titres in Sydenham's chorea. Pediatrics 1966 Dec; 38 (6): 946-956.

Ayoub EM, Shulman ST. Pattern of antibody response to the streptococcal group A carbohydrate in rheumatic patients with or without carditis. In: Read SE, Zabriskie JB(eds). Streptococcal Disease and the Immune response. New York: Academic Press; 1980; 649-659.

Ayoub EM. Resurgence of rheumatic fever in the United States: The changing picture of a preventable illness. Postgrad. Med 1992 Sep 1;1;92(3):133-136.

Ayoub EM, Majeed HA. Poststreptococcal reactive arthritis. Curr Opin Rheumatol 2000 Jul ;12(4):306-310.

Bahr GM, Yousof AM, Behbehani K, Majeed HA, Sakkalah S, Souan K, Jarrad I, Geoffrey C, Alouf JE. Antibody levels and in vitro lymphoproliferate responses to streptococcus pyogenes erythrogenic toxin A and mitogen of patients with rheumatic fever. J Clin Microbiol. 1991 Sept; 29 (9): 1789-1794.

Balasa B, Deng C, Lee J, Bradley LM, Dalton DK, Christadoss P, Sarvetnick N. Interferon gamma (INF-gamma) is necessary for the genesis of acetylcholine receptor-induced clinical experimental autoimmune myasthenia gravis in mice. J Exp Med 1997 Aug 4;186 (3):385-391.

Balasa B, Deng C, Lee J, Christadoss P, Sarvetnick N. The Th2 cytokine IL-4 is not required for the progression of antibody-dependant autoimmune myasthenia gravis. J Immunol 1998 Sept 15;161 (6):2856-2862.

Bangham CR. The immune response to HTLV-1. Curr Opin Immunol 2000 Aug; 12 (4): 397-402.

Barnham M. Invasive streptococcal infections in the era before the acquired immune deficiency syndrome: a 10 years' complication of patients with streptococcal bacteraemia in North Yorkshire. J Infect Dis 1989 May; 18 (3): 231-248.

Baumgardner T, Singer HS, Denckla MB, Rubin MA, Abrams MT, Colli MJ, Reiss AL. Corpus callosum morphology in children with Tourette syndrome and attention deficit hyperactivity disorder. Neurology 1996 Aug; 47(2): 477-482.

Beachey EH, Seyer JM, Kang AH. Primary structure of protective antigens of type 24 streptococcal M protein. J. Biol Chem 1980 Jul 10; 255 (13): 6284-6289.

Beachey EH, Seyer JM, Dale JB, Simpson WA, Kang AH. Type specific protective immunity evoked by synthetic peptide of Streptococcus pyogenes M protein. Nature 1981 Jul 30; 292 (5822): 457-459.

Beall B, Facklam R, Hoenes T, Schwartz B. Survey of *emm* gene sequences and T-antigen types from systemic *Streptococcus pyogenes* infection isolates collected in San Francisco, California; Atlanta, Georgia; and Connecticut in 1994 and 1995. J Clin Micriobiol 1997 May; 35 (5): 1231-1235.

Beall B, Fackman RR, Elliott A, Franklin AR, Hoenes T, Jackson D, Laclaire L, Thompson T, Viswanathan. Streptococcal *emm* types associated with T-agglutination types and the use of conserved *emm* gene restriction fragment patterns for subtyping group A streptococci. J Med Microbiol 1998 Oct; 47 (10): 893-898.

Beresford OD, Graham AM. Chorea Gravidarum. J Obstet & Gynae Brit Emp 1950 Aug; 57 (4): 616-625.

Berger T, Rubner P, Schautzer F, Egg R, Ulmer H, Mayringer I, Dilitz E, Deisenhammer F, Reindl M. Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med 2003 Jul 20; 349(2): 139-145.

Berggard K, Johnson E, Morfeldt E, Persson J, Stalhammar-Carlemalm M, Lindahl G. Binding of human C3BP to the hypervariable region of M protein: a molecular mechanism of phagocytosis resistance in *streptococcus pyogenes*. Mol Microbiol 2001 Oct: 42 (2): 539-551.

Bernal F, Graus F, Pifarre A, Saiz A, Benyahia B, Ribalta T. Immunohistochemical analysis of anti-Hu associated paraneoplastic encephalomyelitis. Acta Neuropathol (Berl). 2002 May; 103(5): 509-15.

Bernard P, Bedane C, Mounier M, Denis F, Catanzano G, Bonnetblanc JM. Streptococcal cause of erysipelas and cellulitis in adults. A microbiological study using a direct immunofluorescence technique. Arch Dermatol 1989 Jun; 125 (6): 779-782.

Bhakdi S, Roth M, Sziegoleit A, Tranum-Jensen J. Isolation and identification of two haemolytic forms of streptolysin-O. Infect Immun 1984;46: 394-400.

Bhakdi S, Tranum-Jensen J, Sziegoleit A. Mechanism of membrane damage by streptolysin O. Infect Immunol 1985 Jan; 47(1): 52-60

Bhatia KP, Marsden CD. The behavioural and motor consequences of focal lesions in the basal ganglia in man. Brain 1994 Aug;117 (pt 4); 859-876.

Bhatia R, Narula J, Reddy KS, Koicha M, Malaviya AN, Pothineni RB, Tandon R, Bhatia ML. Lymphocyte subsets in acute rheumatic fever and rheumatic heart disease. Clin Cardiol. 1989 Jan; 12(1): 34-38.

Bisno AL, Brito MO, Collins CM. Molecular basis of group A streptococcal virulence. Lancet Infect Dis 2003 Apr; 3(4): 191-200.

Bodner SM, Morshed SA, Peterson BS. The question of PANDAS in adults. Biol Psychiatry 2001 May 1; 49(9):807-10.

Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997;15: 749-795.

Bonora G, Rogari P, Acerbi L, Frattini D, Perletti L. Outbreak of acute rheumatic fever in northern Italy. J Pediatr 1989 Feb;114(2):334-335.

Bouteile EM. Traite de la Choree, ou Danse de St. Guy. Paris, Vincard; 1810

Bouyer JJ, Park DH, Joh TH, Pickel VM. Chemical and structural analysis of the relationship between cortical inputs and tyrosine hydroxylase-containing terminals in rat neostriatum. Brain Res 1984 Jun; 302(2): 267-275.

Breese BB. A simple scorecard for the tentative diagnosis of streptococcal pharyngitis. Am J Dis Child 1977 May;131 (5): 514-517.

Bremm KD, Konig W, Thelestam M, Alouf JE. Modulation of granulocyte functions by bacterial exotoxins and endotoxins. Immunol 1987 Nov; 62 (3): 363-371.

Bronze MS, Dale JB. Epitopes of streptococcal M proteins that evoke antibodies that cross-react with Human brain. J. Immunol 1993 Sep 1; 151(5): 2820-2828.

Brown J, Bullock D, Grossberg S. How the basal ganglia use parallel excitatory and inhibitory learning pathways to selectively respond to unexpected rewarding cues. J Neurosci 1999 Dec;19 (23): 10502-10511.

Brown JH. The use of blood agar for the study of streptococci. Monogr No 9. new York: Rockefeller institute of Medical Research 1919.

Caparon MG, Stephens DS, Olsen A, Scott JR.Role of M protein in adherence of group A streptococci. Infect Immunol 1991 May; 59 (5): 1811-1817.

Carapetis JR, Currie BJ. Rheumatic chorea in northern Australia: a clinical and epidemiological study. Arch Dis Child 1999 Apr; 80 (4): 353-358.

Cardoso F, Vargas AP, Oliveira LD, Guerra AA, Amaral SV. Persistent Sydenham's chorea. Mov disord 1993 Sep; 14(5):805-807.

Cardoso F, Eduardo C, Silva AP, Mota CC. Chorea in fifty consecutive patients with rheumatic fever. Mov disord 1997 Sep; 12(5):701-703.

Cardoso F. Chorea Gravidarum. Arch Neurol 2002 May; 59 (5):868-70.

Cardoso F, Maia D, Cunningham MC, Valenca G. Treatment of Sydenham chorea with corticosteroids. Mov Disord 2003 Nov; 18(11): 1374-1377.

Cardona F, Orefici G: Group A streptococcal infections and tic disorders in an Italian pediatric population. J.Pediatr. 2001 Jan;138(1):71-5.

Castillo M, Kwock L, Arbelaez A. Sydenham's chorea: MRI and proton spectroscopy. Neuroradiology 1999 Dec;41 (12): 943-945.

Cavelti PA. Autoantibodies in rheumatic fever. Proc Soc Exp Biol Med 1945; 60:379-381.

Cherubini E, Herrling PL, Lanfumey L, Stanzione P. Excitatory amino acids in synaptic excitation of rat striatal neurones in vitro. J Physiol 1998 Jun; 400: 677-690.

Church AJ, Cardoso F, Dale RC, Lees AJ, Thompson EJ, Giovannoni G. Anti-basal ganglia antibodies in acute and persistent Sydenham's chorea. Neurology 2002 Jul 23; 59 (2): 227-231.

a.Church AJ, Dale RC, Cardoso F, Candler PM, Chapman MD, Allen ML, Klein NJ, Lees AJ, Giovannoni G. CSF and serum immune parameters in Sydenham's chorea: evidence of an autoimmune syndrome? J. Neuroimmunol 2003 Mar; 136(1-2): 149-153.

b.Church AJ, Dale RC, Lees AJ, Giovannoni G, Robertson MM. Tourette's syndrome a cross sectional study to examine the PANDAS hypothesis. J Neurol Neurosurg Psych 2003 May; 74 (5): 602-607.

Collin M, Olsen A. Effect of SpeB and EndoS from Streptococcus pyogenes on human immunoglobulins. Infect Immunol 2001 Nov; 69 (11): 7187-7189.

Colony HS, Malamud N. Sydenham's Chorea. A clinicopathologic study. Neurology 1956; 6:672-676.

Copley SD. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. Curr Opin Chem Biol 2003 Apr; 70(2): 265-272.

Cornfield D, Warner G, Weaver R, Bellows MT, Hubbard JP. Streptococcal infection in a school population: preliminary report. Ann Intern Med 1958; 49: 1305-1319.

Cossete M, Levesque M, Parent A. Extrastriatal dopaminergic innervation of human basal ganglia. Neurosci Res 1999 May;34 (1): 51-54.

Creak M, Guttman E. Chorea, tics, compulsive utterances. J Med Sci 1935;81:834

Cue D, Dombek PE, Lam H, Cleary PP. Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells. Infect Immunol 1998 Oct; 66 (10): 4593-4601.

Cu GA, Mezzano S, Bannan JD, Zabriskie JB. Immunohistochemical and serological evidence for the role of streptococcal proteinase in acute post-streptococcal glomerulonephritis. Kidney Int 1998 Sep; 54(3):819-826.

Cunningham MW, Swerlick RA. Polyspecificity of anti-streptococcal murine monoclonal antibodies and their implications in autoimmunity. J Exp Med 1986 Oct 1; 164 (4):998-1012.

Cunningham MW, McCormack JM, Talaber LR, Harley JB, Ayoub EM, muneer RS, Chun LT, Reddy DV. Human monoclonal antibodies reactive with antigens of the group A streptococcus and human heart. J Immunol 1988 Oct 15; 141(8):2760-2766.

Cunningham MW, McCormack JM, Fenderson PG, Ho MK, Beachey EH, Dale JB. Human and murine antibodies cross-reactive with streptococcal M protein and myosin recognize the sequence GLN-LYS-SER-LYS-GLN in M protein. J Immuol 1989 Oct 15;143 (8):2677-2683.

Cunningham MW, Antone SM, Gulizia JM, McManus BM, Fischetti VA, Gauntt CJ. Cytotoxic and viral neutralising antibodies crossreact with streptococcal M protein, enterovirus and human cardiac myosin. Proc Natl Acad Sci 1992 Feb 15; 89 (4):1320-1324.

Cunningham MW, Antone SM, Gulizia JM, McManus BA, Gauntt CJ. Alpha-helical coiled-coil molecules: a role in autoimmunity against the heart. Clin Immul Immunopath 1993 Aug; 68(2): 118-123.

Cunningham MW, Meissner HC, Heuser JS, Pietra BA, Kurahara DK, Leung DY. Anti-human Cardiac Myosin Autoantibodies in Kawasaki Syndrome. J Immunol. 1999 Jul 15; 163(2): 1060-1065.

Cunningham MW. Pathogenesis of Group A Streptococcal infections. Clin Microbiol Rev 2000 Jul;13(3): 470-511.

Cunningham MW. Autoimmunity and molecular mimicry in the pathogenesis of post-streptococcal heart disease. Front Biosci 2003 May 1;8: s533-543.

Dagan R, Ferne M, Sheinis M, Alkan M, Katzenelson E. An epidemic of penicillin-tolerant group A streptococcal pharyngitis in children living in a closed community:mass treatment with erythromycin. J Infect Dis 1987 Sep;156 (3):514-516.

Dajani AS, Ferrieri P, Wannamaker LW. Natural history of impetigo. II. Etiologic agents and bacterial interactions. J Clin Invest 1972 Nov;51 (11):2863-2871.

Dale JB, Beachey EH. Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart. J Exp Med 1982 Oct 1;156 (4): 1165-1176.

Dale JB, Beachey EH. Epitopes of streptococcal M proteins shared with cardiac myosin. J Exp Med 1985 Aug 1;162(2): 582-591.

Dale JB, Beachey EH. Human cytotoxic T lymphocytes evoked by group A streptococcal M proteins. J Exp Med 1987 Dec 1; 166(6):1825-1835.

Dale RC, Church AJ, Cardoso F, Goddard E, Cox TC, Chong WK, Williams A, Klein NJ, Neville BG, Thompson EJ, Giovannoni G. Post-streptococcal acute disseminated encephalomyelitis with basal ganglia involvement and auto-reactive antibasal ganglia antibodies. Ann Neurol 2001 Nov; 50(5): 588-595.

Dale RC, Church AJ, Benton S, Surtees RA, Lees A, Thompson EJ, Giovannoni G, Neville BG. Post-streptococcal autoimmune dystonia with isolated bilateral striatal necrosis. Dev Med Child Neurol 2002 Jul; 44(7): 485-489.

Dale RC. Autoimmunity and the basal ganglia: new insights into old diseases. Q J Med 2003 Mar; 96(3): 183-191.

Dale RC, Church AJ, Surtees RA, Lees AJ, Adcock JE, Harding B, Neville BG, Giovannoni G. Encephalitis lethargica syndrome: 20 new cases and evidence of basal ganglia autoimmunity. Brain 2004 Jan; 127 (pt 1): 21-33.

Dale RC, Candler PM, Church AJ, Wait R, Pocock J, Giovannoni G. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. J Neuroimm 2005 (in press).

Damaschun G, Damaschun H, Gast K, Gerlach D, Misselwitz R, Welfle H, Zirwer D. Streptokinase is a flexible multi-domain protein. Eur Biophys 1992; 20: 355-361.

Darmstadt GL, Lane AT. Impetigo: an overview. Pediatr Dermatol 1994 Dec; 11(4): 293-303.

Davidson A, Diamond B. Autoimmune diseases.New Engl J Med 2001 Aug 2; 345(5): 340-350.

Delcourt & Sand. Archiv de Med des enfants 1908; 11:826.

de Scheerder IK, de Buyzere ML, Delanghe JR, Clement DL, Wieme RJ. Anti-myosin humoral immune response following cardiac injury 1989. Autoimmunity; 4(1-2):51-8

Denny-Brown D. Symposium on disease of the basal ganglia. Nature of dystonia. Rev

Bras Med 1968; 25:769-773.

Derbise A, Song YP, Parikh S, Fischetti VA, Pancholi V. Role of the the C-terminal lysine residues of streptococcal surface enolase Glu- and Lys-plasminogen binding activities of group A streptococci. Infect Immunol 2004 Jan; 72(1): 94-105.

Desai A, Ravi V, Guru SC, Shankar SK, Kaliaperumal VG, Chandramuki A, Gourie-Devi M. Detection of autoantibodies to neural antigens in the CSF of Japanese encephalitis patients and correlation of findings with the outcome. J Neurol Sci 1994 Mar; 122(1): 109-116.

Destée A, Muller JP, Vemersch P, Pruvo JP, Warot P. Hemiballisme. Hemichoée. Infarctus striatal. Rev Neurol Rev Neurol 1990; 146(5):150-152. (French)

Dighiero G, Rose NR. Critical self epitopes are key to the understanding of self-tolerance and autoimmunity. Immunol Today 1999 Sep;20 (9): 423-428.

Dilley A, Gregson NA, Hadden RD, Smith KJ. Effects on axonal conduction of anti-ganglioside sera from patients Guillain-Barre syndrome. J Neuroimmunol 2003 Jun; 139 (1-2): 133-140.

Dinkla K, Rohde M, Jansen WT, Carapetis JR, Chhatwal GS, Talay SR. Streptococcal pyogenes recruits collagen via surface-bound fibronectin: a novel colonization and immune evasion mechanism. Mol Microbiol 2003 Feb; 47(3):861-869.

DiSciascio G, Taranta A. Rheumatic fever in children. Am Heart J 1980 May; 99(5): 635-658.

Dombek PE, Cue D, Sedgewick J, Lam H, Ruschkowski S, Finlay BB, Cleary PP. High frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. Mol Micriobiol 1999 Feb; 31(3): 859-870.

Donadi EA, Smith AG, Louzada-Junior P, Voltarelli JC, Nepom GT. HLA class I and II profiles of patients presenting with Sydenham's chorea. J Neurol 2000 Feb; 247:122-128.

Dropcho EJ. Paraneoplastic Diseases of the Nervous System. Curr Treat Otions Neurol. 1999 Nov; 1(5) 417-427.

Dudding BA, Ayoub EM. Persistence of streptococcal group A antibody in patients with rheumatic valvular disease. J Exp Med. 1968 Nov 1; 128:1081-1098.

Eapen V, Pauls DL, Robertson MM: Evidence for autosomal dominant transmission in Tourette's syndrome. United Kingdom cohort study. Br J Psychiatry 1993 May; 162: 593-596.

Efstratiou A. Outbreaks of human infection caused by pyogenic streptococci of Lancefield groups C and G. J Med Microbiol 1989 Jul; 29(3): 207-219.

Egg R, Reindl M, Deisenhammer F, Linington C, Berger T. Anti-MOG and anti-MBP antibody subclasses in multiple sclerosis. Multiple Sclerosis 2001 Oct; 7(5): 285-289.

Ehlenberger AG, Nussenzweig V. Role of C3b and C3d receptors in phagocytosis. J Exp Med 1977 Feb 1; 145: 357-371.

Ellen RP, Gibbons RJ. M protein associated adherence of Streptococcal pyogenes to epithelial surfaces: prerequisite for virulence. Infec. Immun. 1974 May; 5(5): 826-830.

Ermann J, Fathman GC. Autoimmune diseases: genes, bugs and failed regulation. Nat Immunol 2001 Sep; 2(9): 759-761.

Eshel G, Lahat E, Azizi E, Gross B, Aladjem M. Chorea as a manifestation of rheumatic fever: a 30-year survey (1960-1990). Eur J Pediatr 1993; 152(8): 645-646.

Facinelli B, Spinaci C, Magi G, Giovanetti E, Varado P. Association between erythromycin resistance and ability to enter human respiratory cells in group A streptococci. Lancet 2001 Jul 7; 358 (9275): 30-33.

Fahn S. Concept and classification of dystonia. Adv Neurol 1988; 50: 1-8.

Falcone M, Sarvetnick N. Cytokines that regulate autoimmune responses. Curr Opin Immunol 1999 Dec; 11(6): 670-676.

Falcone M, Yeung B, Tucker L, Rodriguez E, Krahl T, Sarvetnick N. IL-4 triggers autoimmune diabetes by increasing self-antigen presentation within the pancreatic Islets. Clin Immunol 2001 Feb; 98 (2): 190-199.

Faustino PC, Terreri MT, da Rocha AJ, Zappitelli MC, Lederman HM, Hilario MO. Clinical, laboratory, psychiatric and magnetic resonance findings in patients with Sydenham chorea. Neuroradiol 2003; 45(7): 456-462.

Fenderson PG, Fischetti VA, Cunningham MW. Tropomyosin shares immunologic epitopes with group A streptococcal M proteins. J Immunol 1989 Apr 1; 142(7): 2475-2481.

Ferrier D. The functions of the brain. 1876; London (Br Library).

Fillit H, Damle SP, Gregory JD, Volin C, Poon-King T, Zabriskie J. Sera from patients with poststreptococcal glomerulonephritis contains antibodies to glomerular heparin sulfate proteoglycan. J Exp Med 1985 Feb 1; 161(2):277-89.

Fischetti VA. Streptococcal M protein: molecular design and biological behaviour. Clin Microbiol Rev 1989 Jul; 2(3): 258-314

Fischetti VA, Horstmann RD, Pancholi V. Location of the complement factor H binding site on streptococcal M6 protein. Infect Immunol 1995 Jan; 63(1): 149-153

Fontan PA, Pancholi V, Nociari MM, Fischetti VA. Antibodies to Streptococcal surface enolase react with Human alpha-enolase: implications in poststreptococcal sequelae. J Infect Dis 2000 Dec; 182(6): 1712-1721.

Franciosi RA, Knostman JD, Zimmerman RA. Group B streptococcal neonatal and infant infections. J Pediatr 1973 Apr; 82(4): 707-718.

Freeman JM, Aron AM, Collard JE, MacKay MC. The emotional correlates of Sydenham's chorea. Pediatrics 1965 Jan; 35: 42-49.

Friedman I, Laufer A, Ron N, Davies AM. Experimental myocarditis: in vitro and in vivo studies of lymphocytes sensitised to heart extracts and group A streptococci. Immunol 1971 Feb; 20(2): 225-232.

Frikke MJ, Seshi B, Bell CE jr. Monoclonal antibodies to human neuron-specific enolase reveal heterogeneity of the enzyme in neurones of the central nervous system. Brain Res 1987 Aug 11; 417(2): 283-292.

Galvin JE, Hemric ME, Ward K, Cunningham MW. Cytotoxic mAb from rheumatic carditis recognises heart valves and laminin. J Clin Invest 2000 Jul; 106(2): 217-224.

Galvin JE, Hemric ME, Kosanke SD, Factor SM, Quinn A, Cunningham MW. Induction of myocarditis and valvulitis in lewis rats by different epitopes of cardiac myosin and its implications in rheumatic carditis. Am J Pathol 2002 Jan; 160(1): 297-306.

Garvey MA, Perlmutter SJ, Allen AJ, Hamburger S, Lougee L, Leonard HL, Witowski ME, Dubbert B, Swedo SE. A pilot study of penicillin prophylaxis for neuropsychiatric exacerbations triggered by streptococcal infections. Biol Psych 1999 Jun 15; 45(12):1564-1571.

Gerfen CR. The neostriatal mosaic: compartmentalisation of corticostriatal input and striatonigral output systems. Nature 1984 Oct 4-10; 311(5985): 461-464.

Giagheddu M, Tamburini G, Piga M, Tacconi P, Giagheddu A, Serra A, Siotto P, Satta L, Demelia L, Marrosu F. Comparison of MRI, EEG, EP and ECD-SPECT in Wilson's disease. Acta Neurol Scand 2001 Feb;103(2):71-81.

Gibb WR, Lees AJ, Scadding JW. Persistent rheumatic chorea. Neurology 1985 Jan; 35(1):101-102.

Gibofsky A, Kerwar S, Zabriskie JB. Rheumatic fever. The relationships between host, microbe, and genetics. Rheum Dis Clin North Am 1998 May;24(2):237-259.

Giedd JN, Rapoport JL, Kruesi MJ, Parker C, Schapiro MB, Allen AJ, Leonard HL, Kaysen D, Dickstein DP, Marsh WL, Kozuch PL, Vaituzis AC, Hamburger S, Swedo SE. Sydenham's chorea: Magnetic resonance imaging of the basal ganglia. Neurology 1995 Dec; 45(12):2199-2202.

Giedd JN, Rapoport JL, Leonard HL, Richter D, Swedo SE. Case study: acute basal ganglia enlargement and obsessive-compulsive symptoms in an adolescent boy. J Am Acad Child Adolesc Psych 1996 Jul;35(7): 913-915.

Giedd JN, Rapoport JL, Garvey MA, Perlmutter S, Swedo SE. MRI assessment of children with obsessive-compulsive disorder or tics associated with streptococcal infection. Am J Psych 2000 Feb; 157(2): 281-283.

Gilles de la Tourette G. Etude sur une affection nerveuse caracterisee par de l'incoordination motrice accompagnee d'echolalie et de copralalie. Arch Neurol 1885;9 (19-24) :158-200.

Giraudon P, Szymocha R, Buart S, Bernard A, Cartier L, Belin MF, Akaoka H. T lymphocytes activated by persistent viral infection differentially modify the expression of metalloproteinase and their endogenous inhibitors, TIMPs in human astrocytes: Relevance to HTLV-1 induced neurologic disease. J Immunol 2000 Mar 1; 164(5): 2718-2727.

Gladstone P, Jesudason MV, Sridharan G. Invasive properties of south Indian strains of Streptococcus pyogenes in a Hep2 cell model. Clin Microbiol Infect. 2003 Oct; 9(10): 1031-1034.

Glaser GH. Lesions of the central nervous system in disseminated lupus erythematosus. Atch Neurol & Psych. 1952; 67: 745.

Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev 2000 Jul 15;14(14): 1693-1711.

Goldenberg J, Ferraz MB, Hilário MO, Fonseca AS, Bastos W, Sachetti S. Increases in incidence of Sydenham's chorea in São Paulo, Brazil. J Trop Ped 1993 Jun;39(3): 192-193.

Goldman S, Amrom D, Szliwowski HB, Detemmerman D, Goldman S, Bidaut LM, Stanus E, Luxen A. Reversible striatal hypermetabolism in a case of Sydenham's chorea. Mov Dis 1993 Jul; 8(3): 355-358.

Gordon RG, Norman RM. A case of acute toxic chorea. J Neurol & Psychopath. 1935; 15: 313.

Graveland GA, Williams RS, DiFiglia M. Evidence for degenerative and regenerative changes in neostriatal spiny neurones in Huntington's disease. Science 1985 Feb 15; 227(4688): 770-773.

Graybiel AM. Neural networks: neural systems V: basal ganglia. Am J Psych 2001 Jan; 158 (1): 21.

Gray ED, Wannamaker LW, Ayoub EM, el Kholy A, Abdin ZH. Cellular immune responses to extracellular streptococcal products in rheumatic heart disease. J Clin Invest 1981 Sep; 68(3): 665-671.

Green LN. Corticosteroids in the treatment of Sydenham's chorea. Arch. Neurol 1978 Jan; 35(1): 53-54.

Greenfield JG, Wolfsohn JM. The Pathogenesis of Sydenham's chorea. Lancet 1922; 2:603-606.

Gross DM, Forsthuber T, Tary-Lehmann M, Etling C, Ito K, Nagy ZA, Field JA, Steere AC, Huber BT. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis. Science 1998 Jul 31; 281(5377): 703-706.

Guerau-de-Arellano M, Huber BT. Development of autoimmunity in Lyme arthritis. Curr Opin Rheumatol 2002 Jul; 14(4):388-93.

Guilherme L, Cunha-Neto E, Coelho V, Snitcowsky R, Pomerantzeff PMA, Assis RV, Pedra F, Neumann J, Goldberg A, Patarroyo ME, Pileggi F, Kalil J. Human heart-infiltrating T cell clones from rheumatic heart disease patients recognise both streptococcal and cardiac proteins. Circulation 1995 Aug 1; 92(3): 415-420.

Guilherme L, Oshiro SE, Fae KC, Cunha-Neto E, Renesto G, Goldberg AC, Tanaka AC, Pomerantzeff PM, Kiss MH, Silva C, Guzman F, Patarroyo ME, Southwood S, Sette A, Kalil J. T-cell reactivity against streptococcal antigens in the periphery mirrors reactivity of heart-infiltrating T lymphocytes in rheumatic heart disease patients. Infect Immun 2001 Sep; 69(9):5345-51.

Guizzetti & Camisa. Riv Sper de Fernait. E di Med Leg. 1911; 37:266

Gulizia JM, Cunningham MW, McManus BA. Immunoreactivity of anti-streptococcal monoclonal antibodies to human heart valves: Evidence for multiple cross-reactive epitopes. Am J Pathol 1991 Feb; 138(2): 285-301.

Haber SN, Kunishio K, Mizobuchi M, Lynd-Balta E. The orbital and medial prefrontal circuit through the primate basal ganglia. J Neurosci 1995 Jul;15(7 Pt 1): 4851-4867.

Hackett SP, Stevens DL. Streptococcal toxic shock syndrome: synthesis of tumour necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J Infect Dis 1992 May;165(5): 879-885.

Hagman MM, Dale JB, Stevens DL. Comparison of adherence to and penetration of a human laryngeal epithelial cell line by group A streptococci of various M protein types. FEMS Immunol Med Microbiol 1999 Mar; 23(3):195-204.

Haidan A, Talay SR, Rohde M, Sriprakash KS, Currie BJ, Chhatwal GS. Pharyngeal carriage of group C and group G streptococci and acute rheumatic fever in the Aboriginal population. Lancet 2000 Sep; 356 (9236): 1167-1169.

Hallas G. The production of pyrogenic exotoxins by group A streptococci. J Hyg 1985; 95:47-57.

Hallett JJ, Harling-Berg CJ, Knopf PM, Stopa EG, Kiessling LS. Anti-striatal antibodies in Tourette syndrome cause neuronal dysfunction. J Neuroimmunol 2000 Nov;111(1-2): 195-202.

Harbaugh MP, Podbielski A, Hugl S, Cleary PP. Nucleotide substitutions and small scale insertion produce size and antigenic variation in group A streptococcal M1 protein. Mol Microbiol 1993 May; 8(5): 981-991.

Harrison NA, Church A, Nisbet A, Rudge P, Giovannoni G. Late recurrences of Sydenham's chorea are not associated with anti-basal ganglia antibodies. J Neuro Neurosurg Psych 2004; 75: 1478-1479.

Harvier & Levanditi. Bull et Mem de la Soc Med des Hosp de Paris. 1920; xliv., t.iii:583.

Hasty DL, Ofek I, Courtney HS, Doyle RJ. Multiple adhesions of streptococci. Infect Immunol 1992 Jun; 60(6): 2147-2152.

Herdy GV, Zabriskie JB, Chapman F, Khanna A, Swedo SE. Rapid test for the detection of a B-cell marker (D8/17) in Rheumatic fever patients. Braz J Med Biol Res 1992; 25(8): 789-794.

Herrero MT, Hirsch EC, Kastner AM, Ruberg M, Luquin MR, Laguna J, Javoy-Agid F, Obeso JA, Agid. Does neuromelanin contribute to the vulnerability of catecholaminergic neurones in monkeys intoxicated with MPTP? Neurosci 1993 Sep;56(2): 499-511.

Hickey MJ, Granger DN, Kubes P. Molecular mechanisms underlying IL-4 induced leukocyte recruitment in vivo: a critical role for the alpha 4 integrin. J Immunol 1999 Sep 15;163(6): 3441-3448.

Hoekstra PJ, Bijzet J, Limburg PC, Steenhuis M-P, Troost PW, Oosterhoff MD, Korf J, Kallenberg CG, Minderaa RB. Elevated D8/17 expression on B lymphocytes, a marker of Rheumatic Fever, measured with flow cytometry in Tic Disorder patients. Am J Psych 2001 Apr; 158(4): 605-609..

Hoffman KL, Hornig M, Yaddanapudi K, Jabado O, Lipkin WI. A murine model for neuropschiatric disease associated with group A beta-hemolytic streptococcal infection. J Neurosci 2004 Feb 18; 24(7): 1780-1791.

Hornse H, Banerjee S, Zeitlin H, Robertson M: The prevalence of Tourette syndrome in 13-14 year-olds in mainstream schools. J Child Psychol Psychiat 2001 Nov; 42(8): 1035-1039.

Horstmann RK, Sievertsen HJ, Leippe M, Fischetti VA. Role of fibrinogen in complement inhibition by streptococcal M protein. Infect Immunol 1992; 60: 5036-5041.

Humphrey JH, Pagel WB. The tissue response to heat killed streptococci in the skin of normal subjects and in persons with rheumatic fever, rheumatoid arthritis, subacute bacterial endocarditis and erythema nodosum. Br J Exp Pathol. 1949; 30: 282-288

Husby G, Van de Rijn I, Zabriskie JB, Abdin ZH, Williams RC Jr. Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. J. Exp. Med 1976 Oct 1; 144(4):1094-1110.

Igari J, Kosakai N, Shibazaki S. Antideoxyribonuclease B distribution in healthy individuals. Jpn Circ J 1983 Nov; 47(11):1287-1289.

Iglesias-Gamarra A, Mendez EA, Cuellar ML, Ponce de Leon JH, Jimenez C, Canas C, Restrepo J, Pena M, Valle R, Espinoza LR. Poststreptococcal reactive arthritis in adults: long-term follow-up. Am J Med Sci 2001 Mar; 321(3):173-177.

Inuzuka T. Autoantibodies in paraneoplastic neurological syndrome. Am J Med Sci. 2000; 319 (4): 217-26.

Ishak R, Cavalcante F, Vallinoto AC, Azevedo VN, Ishak MO. HTLV-1 associated myelopathy in the northern region of Brazil (Belem-Para): serological and clinical features of three cases. Rev Soc Med Trop 2002 May-Jun; 35(3):243-246.

Izumo S, Umehara F, Osame M. HTLV-1 associated myelopathy. Neuropath 2000 Sep; 20 suppl: S65-8

Jacobson S, Shida H, McFarlin DE, Fauci A, Koenig S. Circulating CD8+ cytotoxic lymphocytes specific for HTLV-1 pX in patients with HTLV-1 associated neurological disease. Nature 1990 Nov; 348(6298): 245-248.

Jankovic J, Ford J. Blepharospasm and orofacial-cervical dystonia: clinical and pharmacological findings in 100 patients. Ann Neurol 1983 Apr; 13(4): 402-411.

Jankovic J: Tourette's syndrome. N.Engl.J.Med. 2001 Oct; 345(16):1184-1192.

Jason J, Gregg L, Han A, Hu A, Inge KL, Eick A, Tham I, Campbell R. Immunoregulatory changes in Kawasaki disease. Clin Immunol Immunopath 1997 Sep; 84(3): 296-306.

Jernigan M, Morcos Y, Lee SM, Dohan FC Jr, Raine C, Levin MC. IgG in brain correlates with clinicopathological damage in HTLV-1 associated neurological disease. Neurology 2003 Apr 22; 60(8): 1320-1327.

Johnsson E, Berggard K, Kotarsky H, Hellwage J, Zipfel PF, SjÖbring U, Lindahl G. Role of the hypervariable region in streptococcal M proteins: binding of human complement Inhibitor. J Immunol 1998 Nov 1;161(9): 4894-4901.

Jones TD, Bland EF. Clinical significance of chorea as a manifestation of rheumatic fever: a study in prognosis. JAMA 1935; 105: 571-577.

Jones TD. The diagnosis of rheumatic fever. JAMA. 1944; 126:481-484.

Jones KF, Fischetti VA. The importance of the location of antibody binding on the M6 protein for opsonisation and phagocytosis of group A M6 streptococci. J Exp Med 1988 Mar 1; 167(3): 1114-1123.

Joseph J, Cruz-Sanchez FF, Carreras J. Enolase activity and isoenzyme distribution in human brain regions tumours. J Neurochem 1996; 66 (6): 2484-2490.

Jummani R, Okun M. Sydenham Chorea. Arch Neurol 2001 Feb; 58(2):311-313.

Kadesjo B, Gillberg C: Tourette's disorder: epidemiology and comorbidity in primary school children. J Am Acad Child Adolesc Psych 2000 May; 39(5): 548-555

Kantor FS. Fibrinogen precipitation by streptococcal M protein: Identity of the reactants and stoichiometry of the reaction. J Exp Med 1965 May 1; 121: 849-859.

Kaplan MH, Bolande R, Rakita L, Blair J. Presence of bound immunoglobulins and complement in the myocardium in acute rheumatic fever. Association with cardiac failure. N Engl J Med 1964 Sep 24; 271:637-645.

Kaplan EL, Ferrieri P, Wannamaker LW. Comparison of the antibody response to streptococcal cellular and extracellular antigens in acute pharyngitis. J Pediatr. 1974 Jan; 84(1): 21-28.

Kaplan EL, Wannamaker LW. Streptolysin O: suppression of its of its antigenicity by lipids extracted from skin. Proc Soc Exp Biol Med 1974 May;146(1): 205-208.

Kaplan EL, Rothermel CD, Johnson DR. Antistreptolysin O and antideoxyribonuclease B titers: normal values for children ages 2 to 12 in the United States. Pediatrics 1998 Jan;101 (Pt 1): 86-88.

Kato K, Asai R, Shimizu F, Suzuki F, Ariyoshi Y. Immunoassay of three enolase isozymes in human serum and in blood cells. Clin Chim Acta 1983 Feb 7; 127(3): 353-363.

Keefer CS, Ingelfinger FJ, Spink WW. Significance of hemolytic streptococci bacteremia; a study of two hundred and forty-sex patients. Arch Intern Med 1937; 60:1084-1097

Kemeny E, Grieve T, Marcus R, Sareti P, Zabriskie JB. Identification of mononuclear cells and T cell subsets in rheumatic valvulitis. Clin Immunol Immunopathol 1989 Aug; 52(2): 225-237.

Kerbeshian J, Burd L, Pettit R. A possible post-streptococcal movement disorder with chorea and tics. Dev Med Child Neurol 1990 Jul; 32(7): 642-644.

Khandke KM, Fairwell T, Acharya AS, Manjula BN. Domain structure and molecular flexibility of streptococcal M protein in situ probed by limited proteolysis. J Prot Chem 1990 Oct; 9(5): 511-522.

Khanna AK, Buskirk DR, Williams RC, Gibofsky A, Crow MK, Menon A, Fotino M, Reid HM, Poon-King T, Rubinstein P, Zabriskie JB. Presence of a non-HLA B cell antigen in rheumatic fever patients and their families as defined by a monoclonal antibody. J Clin Invest 1989 May; 83(5): 1710-1716.

Khanna AK, Nomura Y, Fischetti VA, Zabriskie JB. Antibodies in the sera of acute rheumatic fever patients bind to human cardiac tropomyosin. J Autoimmun 1997 Feb;10(1):99-106.

Kienzle GD, Breger RK, Chun RWM, Zupanc ML, Sackett JF. Sydenham chorea: MR manifestations in two cases. Am J Neuroradiol 1991 Jan-Feb;12(1) :73-76.

Kiessling LS, Marcotte AC, Culpepper L. Anti-neuronal antibodies in movement disorders. Pediatrics 1993 Jul; 92 (1):39-43.

Kirch DG. Infection and autoimmunity as etiologic factors in schizophrenia: a review

and reappraisal. Schizophren Bull. 1993; 19(2): 355-70.

Kirvan CA, Swedo SE, Heuser JS, Cunningham MW. Mimicry and autoantibody-mediated neuronal cell signalling in Sydenham chorea. Nat med 2003 Jul; 9(7): 914-920.

Klein GC, Baker CN, Jones WL. "Upper limits or normal" antistreptolysin O and antideoxyribonuclease B titres. Appl Microbiol 1971 Jun; 21(6): 999-1001.

Kodama M, Matsumoto Y, Fujiwara M, Massani F, Izumi T, Shibata A. A novel experimental model of giant cell myocarditis induced in rats by immunization with cardiac myosin fraction. Clin Immunol Immunopathol 1990 Nov; 57(2) :250-262.

Kotby AA, El Badawy N, El Sokkary S, Moawad H, Shawarby M. Antineuronal Antibodies in Rheumatic Chorea. Clin. Diag. Lab. Immunol 1998 Nov; 5(6):836-839.

Krisher K, Cunningham MW. Myosin: a link between streptococci and heart. Science 1985 Jan 25; 227(4685): 413-415.

Kulkarni ML, Anees S. Sydenham's chorea. Indian Pediatr 1996 Feb; 33(2):112-5.

Kuo CF,Wu JJ, Tsai PJ, Kao FJ, Lei HY, Lin MT, Lin YS. Streptococcal pyrogenic exotoxin B induces apoptosis and reduces phagocytic activity in U937 cells. Infect immune 1999 Jan; 67(1): 126-130.

Kurlan R, Behr J, Medved L, Shoulson I, Pauls D, Kidd JR, Kidd KK: Familial Tourette's syndrome: report of a large pedigree and potential for linkage analysis. Neurology 1986 Jun; 36(6): 772-776.

Kurlan R. Tourette's syndrome and 'PANDAS': will the relationship bear out? Pediatric Autoimmune neuropsychiatric disorders associated with streptococcal infection. Neurology 1998 Jun; 50(6):1530-1534.

Kurlan R, McDermott MP, Deeley C, Como PG, Brower C, Eapen S, Andresen EM, Miller B: Prevalence of tics in schoolchildren and association with placement in special education. Neurology 2001 Oct 23; 57(8):1383-1388.

Lai RB, Giam CZ, Coligan JE, Rudolph DL. Differential immune responsiveness to the immunodominant epitopes of regulatory proteins (tax and rex) in human T-cell lymphotropic virus type I-associated myelopathy. J Infect Dis 1994 Mar;169(3): 496-503.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970 Aug 15; 227(259):680-685.

Lancefield RC. A serological differentiation of human and other groups of hemolytic streptococci. J Exp Med 1933;57:571-595

Lancefield RC. Current knowledge of the type and specific M antigens of group A streptococci. J Immunol 1962 Sep; 89: 307-313.

Lang B, Pinto A, Giovannini F, Newsom-Davis J, Vincent A. Pathogenic autoantibodies in the lambert-eaton myasthenic syndrome. Ann N Y Acad Sci 2003 Sep; 998: 187-195.

LaPenta D, Rubens C, Chi E, Cleary PP. Group A streptococci efficiently invade human respiratory epithelial cells. Proc Natl Acad Sci USA 1994 Dec 6; 91(25): 12115-12119.

Laplane D, Levasseur M, Pillon B, Dubois B, Baulac M, Mazoyer B, Tran Dinh S, Sette G, Danze F, Baron JC. Obsessive-compulsive and other behavioural changes with bilateral basal ganglia lesions. A neuropsychological, magnetic imaging and positon tomography study. Brain 1989 Jun;112 (Pt 3): 699-725.

Lassek AM. The human pyramidal tract. II. A numerical investigation of the Betz cells of the motor area. Arch Neurol Psych 1940;44: 718-724.

Laurino JP, Hallett J, Kiessling LS, Benson M, Pelletier T, Kuhn C. An Immunoassay for anti-neuronal antibodies associated with involuntary repetitive movemment disorders. Ann Clin Lab Sci 1997 May-Jun;27(3): 230-235.

Leckman JF, Riddle MA, Hardin MT, Ort SI, Swartz KL, Stevenson J, Cohen DJ. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. J Am Acad Child Adolesc Psych 1989 Jul; 28(4): 566-573.

Leckman JF, Zhang H, Vitale A, Lahnin F, Lynch K, Bondi C, Kim YS, Peterson BS. Course of tic severity in Tourette syndrome: the first two decades. Pediatr 1998 Jul;102 (1 Pt1):14-19.

Lee PH, Nam HS, Lee KY, Lee BI, Lee JD. Serial brain spect images in a case of Sydenham' s chorea. Arch Neurol 1999 Feb; 56(2): 237-240.

Léger GC, Johnson N, Horowitz SW, Dale RC, Church AJ, Mesulam MM. Dementia-Like presentation of Striatal hypermetabolic state with antistriatal antibodies responsive to steroids. Arch Neurol 2004, May; 61(5): 754-757.

Lehoczkya TV. Ueber die Gehirnveranderungen bei der chorea minor (Sydenham). Arch Psych. 1941;113: 463.

Leonard BA, Lee PK, Jenkins MK, Schlievert PM. Cell and receptor requirements for streptococcal pyrogenic exotoxin T-cell mitogenicity. Infec Immun 1991 Mar; 59(3): 1210-1214.

Leonard HL, Swedo SE. Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS). Int J Neuropsychopharmacol 2001 Jun; 4(2):191-198.

Leung, TK, Hall C, Monfries C, Lim L. Trifluoroperazine activates and releases latent ATP-generating enzymes associated with the synaptic plasma membrane. J Neurochem 1987 49 (1): 232-238

Levin MC, Lee SM, Kalune F, Morcos Y, Dohan FC Jr, Hasty KA, Callaway JC, Zunt J, Desiderio D, Stuart JM. Autoimmunity due to molecular mimicry as a cause of neurological disease. Nat Med 2002 May; 8(5): 509-513.

Lewy, FH. Die hitopathologie der choreatischen Erkrankungen. Ztschr. Neurol.u.Pschiat. 1923; 85: 632.

Lhermitte J, Pagniez P. Anatomie et Physiologie Pathologiques de la choree de Sydenham. Enceph. 1930; 25: 24.

Lim L, Hall C, Leung T, Mahadevan L, Whatley S. Neurone-specific enolase and creatine phosphokinase are protein components of rat brain synaptic plasma membranes. J Neurochem 1983 Oct; 41(4): 1177-1182.

Loiselle CR, Lee O, Moran TH, Singer HS. Striatal microinfusion of Tourette syndrome and PANDAS sera: failure to induce behavioral changes 2004 Apr; 19(4): 390-396.

Manjula BN, Trus, BL, Fischetti VA. Presence of two distinct regions in the coiled-coil structure of the streptococcal PepM5 protein: relationship to mammalian coiled-coil proteins and implications to its biological properties. Proc Natl Acad Sci USA 1985 Feb; 82(4): 1064-1068.

Marboe CC, Knowles DM 2nd, Weiss MB, Fenoglio JJ Jr. Monoclonal antibody identification of mononuclear cells in endomyocardial biopsy specimens from a patient with rheumatic carditis. Hum Pathol 1985 Apr; 16(4): 332-338.

Marie P, Tretiakoff C. Examen histologique de centres nerveux dans un cas de choree aigue de Sydenham. Rev Neurol 1920; 36:603-6.

Marrack P, Kappler J. The staphyloccal enterotoxins and their relatives. Science 1990 Jun 1; 240(4959): 1066.

Martinelli P, Ambrosetto G,.Minguzzi E, Battaglia S, Rizzo G, Scaglione C. Late-onset PANDAS syndrome with abdominal muscle involvement. Eur Neurol 2002; 48(1):49-51.

Matsell DG, Wyatt RJ, Gaber LW. Terminal complement complexes in acute poststreptococcal glomerulonephritis. Pediatr Nephrol 1994 dec; 8(6): 671-676.

Matsuzaki T, Nakagawa M, Nagai M, Usuku K, Higuchi I, Arimura K, Kubota H, Izumo S, Akiba S, Osame M. HTLV-1 proviral load correlates with progression of motor

disability in HAM/TSP: analysis of 239 HAM/TSP patients including 64 patients followed up for 10 years. J Neurovirol 2001 Jun; 7(3): 228-234.

McLean BN, Luxton RW, Thompson EJ. A study of immunoglobulin G in the cerebrospinal fluid of 1007 patients with suspected neurological disease unsing isoelectric focusing and the Log IgG-Index. A comparison and diagnostic applications. Brain 1990 Oct; 113(pt5): 1269-1289.

Medina E, Goldmann O, Toppel AW, Chhatwal GS. Survival of Streptococcus pyogenes within host phagocytic cells: a pathogenic mechanism for persistence and systemic invasion. J Infect Dis 2003 Feb 15; 187(4): 597-603.

Meiselas LE, Zingale SB, Lee SL, Richman S, Siegel M. Antibody production in rheumatic diseases: The effect of brucella antigen. J Clin Invest. 1961 Oct; 40: 1872-1881.

Mercadante MT, Busatto GF, Lombroso PJ, Prado L, Rosario-Campos MC, de Valle R, Marques-Dias MJ, Kiss MH, Leckman JF, Miguel EC. The psychiatric symptoms of rheumatic fever. Am J Psych 2000 Dec;157(12): 2036-2038.

Mertens NM, Galvin JE, Adderson EE, Cunningham MW. Molecular analysis of cross-reactive anti-myosin/anti-streptococcal mouse monoclonal antibodies. Mol Immunol 2000 Oct; 37(15):901-913.

Molinari G, Talay SR, Valentin-Weigand P, Rhode M, Chhatwal GS. The fibronectin-binding protein of streptococcus pyogenes, SfbI, is involved in the internalization of group A streptococci by epithelial cells. Infect Immunol 1997 Apr; 65(4): 1357-1363.

Money A. Some statistics on of chorea. Brain 1883; 5: 511-520.
Monneaux F, Muller S. Epitope spreading in Systemic Lupus Erythematosus. Arth & Rheum 2002; 46 (6): 1430-1438.

Moore G, Traugott U, Scheinberg L, Raine C. Tropical spastic paraparesis: a model of virus –induced cytotoxic T-cell mediated demyelination? Ann Neurol 1989;26: 523-530.

Moore DP. Neuropsychiatric aspects of Sydenham's chorea: a comprehensive review. J Clin Psych 1996 Sep; 57(9): 407-414.

Moriarty J, Varma A, Stevens J, Fish M, Trimble M, Robertson MM. A volumetric MRI study of Gilles de la Tourette's syndrome. Neurology 1997Aug; 49(2): 410-415.

Morris K, Mohan C, Wahi PL, Anand IS, Ganguly NK. Increase in activated T cells and reduction in suppressor/cytotoxic T cells in acute rheumatic fever and active rheumatic heart disease: a longitudinal study. J Infect Dis 1993 Apr; 167(4): 979-983.

Morshed SA, Parveen S, Leckman JF, Mercadante MT, Bittencourt Kiss MH, Miguel EC, Arman A, Yazgan Y, Fujii T, Paul S, Peterson BS, Zhang H, King RA, Scahill L, Lombroso PJ. Antibodies against neural, nuclear, cytoskeletal, and streptococcal epitopes in children and adults with Tourette's syndrome, Sydenham's chorea, and autoimmune disorders. Biol Psychiatry. 2001 Oct 15; 50(8):566-77.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. Definition according according to profiles of lymphokine activities and secreted proteins. J Immunol 1986 Apr 1;136(7): 2348-2357.

Mosmann TR, Coffman RL. TH1 and TH2 different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7: 145-173.

Motomura M, Lang B, Johnston I, Palace J, Vincent A, Newsom-Davis J. Incidence of serum anti-P/O-type and anti-N-type calcium channel autoantibodies in the Lambert-Eaton myasthenic syndrome. J Neuro Sci 1997 Mar 20; 147(1): 35-42.

Muller N, Riedel M, Straube A, Gunther W, Wilske B. Increased anti-streptococcal antibodies in patients with Tourette's syndrome. Psychiatry Res 2000 Apr 24; 94(1) :43-49.

Muller N, Kroll B, Schwarz MJ, Riedel M, Straube A, Lutticken R, Reinert RR, Reineke T, Kuhnemund O: Increased titers of antibodies against streptococcal M12 and M19 proteins in patients with Tourette's syndrome. Psychiatry Res. 2001 Mar 25; 101(2): 187-193.

Murphy TK, Goodman WK, Fudge MW, Williams R Jr, Ayoub EM, Dalal M, Lewis MH, Zabriskie JB. B Lymphocyte Antigen D8/17: a peripheral marker for childhood-onset Obsessive-Compulsive Disorder and Tourette Syndrome? Am J Psych 1997 Mar;154(3): 402-411.

Murphy TK, Goodman WK, Ayoub EM, Voeller KK. On defining Sydenham's chorea: where do we draw the line? Biol Psych 2000 May; 47(10): 851-857.

Murphy ML, Pichichero ME. Prospective identification and treatment of children with pediatric autoimmune neuropsychiatric disorder associated with group A streptococcal infection (PANDAS). Arch Pediatr Adolesc Med 2002 Apr;156(4): 356-361.

Nagai M, Yamano Y, Brennan MB, Mora CA, Jacobson S. Incerased HTLV-1 proviral load and preferntial expansion of HTLV-1 Tax-specific CD8+ cells in cerebrospinal fluid from patients with HAM/TSP. Ann Neurol 2001 Dec; 50(6): 807-812.

Nakano K. Neural circuits and topographic organisation of the basal ganglia and related regions. Brain Dev 2000 Sep; 22 Suppl 1: S5-S16.

Nausieda PA, Grossman BJ, Koller WC, Weiner WJ, Klawans HL. Sydenham chorea: an update. Neurology 1980 Mar; 30(3): 331-334.

Nausieda PA, Bieliauskas LA, Bacon LD, Hagerty M, Koller WC, Glantz RN. Chronic dopaminergic sensitivity afer Sydenham's chorea. Neurology 1983 Jun; 33(6): 750-754.

Natanson S, Sela S, Moses A, Musser J, Caparon M, Hanski E. Distribution of fibronectin binding proteins among group A streptococci of different M types. J Infect Dis 1995 Apr; 171(4): 871-878.

Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signalling mechanisms and biologic functions. Annu Rev Immunol 1999;17: 701-738.

Neu N, Rose NR, Beisel KW, Herskowitz A, Gurri-Glass G, Craig SW. Cardiac myosin induces myocarditis in genetically predisposed mice. J Immunol 1987 Dec 1; 139(11): 3630-3636.

Neu N, Ploier B, Ofner C. Cardiac myosin induced myocarditis. Heart antibodies are not involved in the induction of the disease. J Immuol 1990 Dec 15; 145(12): 4094-4100.

Newsom-Davis J. Paraneoplastic neurological disorders. J Roy Coll Physicians Lond. 1999 May-Jun; 33(3): 225-227.

Nissenson AR, Baraff LJ, Fine RN, Knutson DW. Postreptococcal acute glomerulonephritis: Fact and controversy. Ann Intern Med 1979 Jul; 91(1):76-86.

Nordstrand A, Norgren M, Holm SE. An experimental model for acute poststreptococcal glomerulonephritis in mice. APMIS 1996 Nov;104(11): 805-816.

Nordstrand A, Norgren M, Ferretti JJ, Holm SE. Streptokinase as a mediator of acute post-streptococcal glomerulonephritis in an experimental mouse model. Infect Immun 1998 Jan; 66(1): 315-321.

Nordstrand A, Norgren M, Holm SE. Pathogenic mechanism of acute post-streptococcal glomerulonephritis. Scand J Infect Dis 1999;31(6):523-537.

Odaka M, Koga M, Yuki N, Susuki K, Hirata K. Longitudinal changes in anti-ganglioside antibodies before and after Guillain-Barre syndrome onset subsequent to Campylobacter jejuni enteritis. J Neuro Sci 2003 Jun 15; 210(1-2): 99-103.

O' Hanlon GM, Plomp JJ, Chakrabarti M, Morrison I, Wagner ER, Goodyear CS, Yin X, Trapp BD, Conner J, Molenaar PC, Stewart S, Rowan EG, Willison HJ. Anti-GQ1b ganglioside antibodies mediate complement-dependant destruction of the motor nerve terminal. Brain. 2001 May; 124(pt5): 893-906.

Okada N, Tatsuno I, Hanski E, Caparon M, Sasakawa C, Streptococcal pyogenes protein F promotes invasion of HeLa cells. Microbiology 1998 Nov;144 (Pt11): 3079-3086.

Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M. HTLV-1 associated myelopathy: a new clinical entity. Lancet 1986 May 3; 1(8488): 1031-1032.

O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. Nat Rev Immunol 2002 Jan; 2(1): 37-45.

Osterland A, Engstrand L. Intracellular penetration and survival of Streptococcus pyogenes in respiratory epithelial cells in vitro. Acta Otolaryngol 1995;115: 685-688.

Osterlund A, Engstrand L. An intracellular sanctuary for Streptococcus pyogenes in human tonsillar epithelium-studies of asymptomatic carriers and in vitro cultured biopsies. Acta Otolaryngol 1997 Nov; 117(6): 883-888.

Palmer M, Vulicevic I, Saweljew P, Valeva A, Kehoe M, Bhakdi S. Streptolysin O: a proposed model of allosteric interaction between a pore forming protein and its target lipid bilayer. Biochem 1998 Feb; 37(8): 2378-2383.

Pancholi V, Fischetti VA. Glyceraldehyde-3-phosphate-dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc Natl Acad Sci 1993 Sep 1; 90(17): 8154-8158.

Pancholi V, Fischetti VA. A-Enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic Streptococci. J Biol Chem 1998 Jun 5; 273(23): 14503-14515.

Pancholi V. Multifunctioal alpha-enolase: its role in diseases. Cell Mol Life Sci 2001; 58 (7): 902-920.

Parent A, Hazrati LN. Anatomical aspects of information processing in primate basal ganglia. Trends Neurosci 1993 Mar;16(3): 111-116.

Parra G, Platt JL, Falk RJ, Rodriguez-jturbe B, Michael AF. Cell populations and membrane attack complex in glomeruli of patients with post-streptococcal glomerulonephritis: identification using monoclonal antibodies by indirect immunofluorescence. Clin Immunol Immunopath 1984 Dec; 33(3): 324-332.

Patton WF, Chung-Welch N, Lopez MF, Cambria RP, Utterback BL, Skea WM. Tris-tricine and Tris-borate buffer systems provide better estimates of human mesothelial cell intermediate filament protein molecular weights than the standard Tris-glycine system. Ann biochem 1991 Aug; 197(1): 25-33.

Peake PW, Pussell BA, Karplus TE, Riley EH, Charlesworth JA. Post-streptococcal glomerulonephritis: studies on the interaction between nephritis strain-associated protein (NSAP), complement and the glomerulus. APMIS 1991 May; 99(5): 460-466.

Penney TJ, Martin DR, Williams LC, de Malmanche SA, Bergquist PL. A single emm gene-specific oligonucleotide probe does not recognise all members of the streptococcus pyogenes M type 1. FEMS Micriobiol Lett 1995 Aug 1;130(2-3): 145-149.

Percheron G, Fenelon G, Leroux-Hugon V, Feve A. History of the basal ganglia system. Slow development of a major cerebral system. Rev Neurol 1994 Aug-Sept;150(8-9): 543-554. (French)

Pergami P, Arbustini E, Butti G, Karau J, Aguzzi A, Poloni TE, Montalbetti L, Egitto MG, Simonetti F, Savoldi F, Ceroni M. Disseminated encephalitis following streptococcal infection. Ital J Neurol Sci 1996 Feb;17(1):87-91.

Perlmutter SJ, Leitman SF, Garvey MA, Hamburger S, Feldman E, Leonard HL, Swedo SE. Therapeutic plasma exchange and intravenous immunoglobulin for obsessive-compulsive disorder and tic disorders in childhood. Lancet 1999 Oct; 354(9185): 1153-1158.

Peter G, Smith AL. Group A streptococci infections of the skin and pharynx. New Eng J Med. 1977 Aug 18; 297(7): 311-317 & 365-370. (Two parts)

Peterson B, Riddle MA, Cohen DJ, Katz LD, Smith JC, Hardin MT, Leckman JF. Reduced basal ganglia volumes in Tourette's syndrome using three-dimensional reconstruction techniques from magnetic resonance images. Neurology 1993 May; 43 (5): 941-949.

Peterson BS, Leckman JF, Tucker D, Scahill L, Staib L, Zhang H, King R, Cohen DJ, Gore JC, Lombroso P. Preliminary findings of antistreptococcal antibody titres and basal ganglia volumes in tic, obsessive-compulsive, and attention deficit/hyperactivity disorders. Arch Gen Psych 2000 Apr; 57(4): 364-372.

Piccolo I, Thiella G, Sterzi R, Colombo N, Defanti CA. Chorea as a symptom of neuroborreliosis:a case study. Ital J Neuro Sci 1998; 19 (4): 235-239.

Poiesz BJ, Poiesz MJ, Choi D. The human T-cell lymphoma/leukaemia viruses. Cancer Invest 2003 Apr; 21(2): 253-277.

Price RA, Kidd KK, Cohen DJ, Pauls DL, Leckman JF. A twin study of Tourette syndrome Arch Gen Psych 1985 Aug; 42(8): 815-820.

Pruksakorn S, Currie B, Brandt E, Phornphutkul C, Hunsakunachai S, Manmontri A, Robinson JH, Kehoe MA, Galbraith A, Good MF. Identification of T cell autoepitopes that cross-react with the C-terminal segment of the M protein of group A streptococci. Int Immunol 1994 Aug;6(8):1235-1244.

Quinn A, Ward K, Fischetti VA, Hemric M, Cunningham MW. Immunological relationship between the class I epitope of streptococcal M protein and myosin. Infect Immun 1998 Sep; 66(9):4418-4424.

Quinn A, Kosanke S, Fischetti VA, Factor SM, Cunningham MW. Induction of autoimmune valvular heart disease by recombinant streptococcal m protein. Infect Immunol 2001 Jun; 69(6): 4072-4078.

Quinn RW, Denny FW, Riley HD. Natural occurrence of haemolytic streptococci in normal school children. Am J Pub Health. 1957 Aug; 47(8):995-1008.

Raizada V, Williams RC Jr, Chopra P, Copinath N, Prakash K, Sharma KB, Cherian KM, Panday S, Arora R, Nigam M, Zabriskie JB, Husby G. Tissue distribution of lymphocytes in rheumatic heart valves as defined by monoclonal anti-T cell antibodies. Am J Med 1983 Jan;74(1): 90-96.

Read FEM, Ciocco A, Taussig HB. Frequency of rheumatic manifestations among siblings, parents, uncles, aunts and grandparents of rheumatic and control patients. Am J Hyg 1938;27:719-737.

Read SE, Fischetti VA, Utermohlen V, Falk RE, Zabriskie JB. Cellular reactivity studies to streptococcal antigens. Migration inhibition studies in patients with streptococcal infections and rheumatic fever. J Clin Invest 1974 Aug; 54(2): 439-450.

Rees JH, Soudain SE, Gregson NA, Hughes RA. Campylobacter jejuni infection and Guillain-Barre syndrome. New Engl J Med. 1995; 333 (21): 1374-9.

Rees JH, Candler P. Paraneoplastic neurological disorders. Cancer Med 2003; 1(3): 78-83.

Reindl M, Linington C, Brehm U, Egg R, Dilitz E, Deisenhammer F, Poewe W, Berger T. Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. Brain 1999 Nov; 122 (Pt11): 2047-2056.

Rejholec V. Incidence of rheumatic fever in relation to immunologic reactivity. Ann Rheum Dis 1957 Mar;16(1): 23-30.

Renneberg J, SÖderstrÖm M, Prellner K, Forsgren A, Christensen P. Age-related variations in anti-streptococcal antibody levels. Eur J Clin Microbiol Infect Dis 1989 Sep; 8(9): 792-795.

Ribak CE, Vaughn JE, Roberts E. The GABA neurons and their axon terminals in rat corpus striatum as demonstrated by GAD immunocytochemistry. J Comp Neurol 1979 Sep 15; 187(2): 261-283.

Rincon J, Viera NT, Romero MJ, Mosquera JA. Increased production of chemotatctic cytokines and elevated proliferation and expression of inercellular adhesion molecule-1 in rat mesangial cells treated with erythrogenic toxin type B and its prescursor isolated from nephritogenic streptococci. Nephrol Dial Transplant 2003 Jun; 18(6):1072-1078.

Robertson MM, Eapen V. The National Hospital interview scheldule for the assessment of Giles de la Tourette syndrome. Int J Methods Psych Res 1996;6: 203-226.

Robertson MM, Banerjee S, Kurlan R, Cohen DJ, Leckman JF, McMahon W, Pauls DL, Sandor P, van de Wetering BJ. The Tourette syndrome diagnostic confidence index: development and clinical complications. Neurology 1999 Dec 10; 53(9): 2108-2112.

Robertson MM: Tourette syndrome, associated conditions and the complexities of treatment. Brain 2000 Mar;123(Pt 3):425-462.

Robertson W, Smith C. Sydenham's chorea in the age of MRI: a case report and review. Pediatr Neurol 2002 Jul; 27(1):65-67.

Robinson JH, Kehoe MA. Group A streptococcal M proteins: virulence factors and protective antigens. Immunol Today 1992 Sep; 13(9): 362-367.

Rolls ET. Neurophysiology and cognitive functions of the striatum. Rev Neurol (Paris)1994 Aug-Sep; 150(8-9): 648-660.

Romagnani S. Human Th1 and Th2 subsets: doubt no more. Immunol Today 1991 Aug;12(8): 256-257.

Romero I, Prevost MC, Perret E, Adamson P, Greenwood J, Couraud PO, Ozden S. Interactions between brain endothelial cells and human T-cell leukaemia virus type I-infected lymphocytes: Mechanisms of viral entry into the central nervous system. J Virol 2000 Jul; 74(13): 6021-6030.

Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today 1993 Sep; 14(9): 426-430.

Rose NR. The role of infection in the pathogenesis of autoimmune disease. Seminars in Immunol 1998 Feb; 10(1): 5-13.

Rosloniec EF, Latham K, GuedezYB. Paradoxical roles of INF-gamma in models of TH1-mediated autoimmunity. Arthrit Res 2002 Jul; 4(6):333-336.

Rotch TM. The hygienic and medical treatment of children. Pediatrics, Philadelphia. Lippincott (British library), 1896.

Royds JA, Parsons MA, Taylor CB, Timperley WR. Enolase distribution in the human brain and its tumours. J Pathol 1982 May; 137(1): 37-49.

Ruckenstein MJ, Mount RJ, Harrison RV. The MRL-lpr/lpr mouse: A potential model of autoimmune inner ear disease. Acta Otolaryngol. 1993 Mar; 113(2): 160-165.

Ruiz N, Wang B, Pentland A, Caparon M. Streptolysin O and adherence synergistically modulate proinflammatory responses of keratinocytes to group A streptococci. Mol Microbiol 1998 Jan; 27(2): 337-346.

Rushdy AA, Cooke RP, Iverson AM, Pickering BJ. Boarding school outbreak of group A streptococcal pharyngitis. Commun Dis Report 1995 Jun 23; 5(7): R106-R108.

Sabbatini A, Dolcher MP, Marchini B, Chimenti D, Moscato S, Pratesi F, Bombardieri S, Migliorino P. Alpha-enolase is a renal-specific antigen associated with kidney involvement in mixed cryoglobulinemia. Clin Exp Rheumatol 1997 Nov-Dec; 15(6): 655-658.

Saouda M, Wu W, Conran P, Boyle MD. Streptocccal pyrogenic exotoxin type B enhances tissue damage initiated by other Streptococcus pyogenes products.J Infect Dis 2001 Sep; 184(6): 723-731.

Sato S, Ono N, Steeber DA, Pisetsky DS, Tedder TF. CD19 regulates B lymphocyte signalling thresholds critical for the development of B-1 lineage cells and autoimmunity. J Immunol 1996 Nov 15; 157(10): 4371-4378.

Scaravilli F, An SF, Groves M, Thom M. The neuropathogenesis of paraneoplastic syndromes. Brain Pathol 1999 Apr; 9(2): 251-260

Scherer MT, Ignatowicz L, Winslow GM, Kappler JW, Marrack P. Superantigens: bacterial and viral proteins that manipulate the immune system. Annu Rev Cell Biol 1993; 9: 101-128.

Sekiya K, Satoh R, Danbara H, Futaesaku Y. A ring shaped structure with a crown formed by streptolysin O on the erythrocyte membrane. J Bacteriol 1993 Sep; 175(18): 5953-5961.

Sekiya K, Danbara H, Yase K, Futaesaku Y. Electron microscopic evaluation of a two step theory of pore formation by streptolysin O. J Baceriol 1996 Dec;178(23): 6998-7002.

Shiffman RN. Guideline maintenance and revision: fifty years of the Jones criteria for diagnosis of rheumatic fever. Arch Pediat Adolesc Med 1995 Jul; 149(7): 727-732.

Shikhman AR, Greenspan AR, Cunningham MW. A subset of mouse monoclonal antibodies cross-reactive with cytoskeletal proteins and group A streptococcal M proteins recognises N-aceytl-beta-D-glucosamine. J Immunol 1993 Oct 1; 151(7): 3902-3914.

Shikhman AR, Cunningham MW. Immunological mimicry between N-acetyl-beta-D-glucosamine and cytokeratin peptides. Evidence for a microbially driven anti-keratin antibody reposnse. J Immunol 1994 May 1; 152(9): 4375-4387.

Shulman ST, Ayoub EM, Victorica BE, Gessner IH, Tamer DF, Hernandez FA. Differences in antibody response to streptococcal antigens in children with rheumatic and non-rheumatic mitral valve disease. Circulation 1974 Dec; 50(6): 1244-1251.

Silverman GJ. B-cell superantigens. Immunol Today 1997 Aug;18(8): 379-386.

Singer HS, Hahn IH, Moran TH. Abnormal dopamine uptake sites in post-mortem striatum from patients with Tourette syndrome Ann Neurol 1991 Oct;30(4): 558-562.

Singer HS, Giuliano JD, Hansen BH, Hallett JJ, Laurino JP, Benson M, Kiessling LS: Antibodies against human putamen in children with Tourette syndrome. Neurology 1998 Jun; 50(6):1618-1624.

Singer HS, Giuliano JD, Hansen BH, Hallett JJ, Laurino JP, Benson M, Kiessling LS. Antibodies against a neuron-like (HTB-10 neuroblastoma) cell in children with Tourette syndrome. Biol Psychiatry. 1999 Sep 15; 46(6):775-780.

Singer HS: Current issues in Tourette syndrome. Mov Disord 2000 Nov; 15(6): 1051-1063.

Singer HS, Loiselle CR, Lee O, Garvey MA, Grus FH. Anti-basal ganglia abnormalities in Sydenham chorea. J Neuroimmunol 2003 Mar; 136(1-2): 154-161.

Smith SC, Allen PM. The role of T cells in myosin-induced autoimmune myocarditis. Clin Immunol Immunopath 1993 Aug; 68(2): 100-106.

Sokol MS. Infection-triggered anorexia nervosa in children: clinical description of four cases. J Child Adolesc Psychopharmacol. 2000; 10(2):133-145.

Spaun J, Bentzon MW, Olesen Larsen S, Hewitt LF. International standard for antistreptolysin O. Bull WHO 1961; 24: 271-279.

Special writing group of the committee of Rheumatic Fever, Endocarditis, and Kawasaki Disease of the Council on Cardiovascular Disease of the Young of the American Heart

Association. Guidelines for the diagnosis of rheumatic fever. J.A.M.A. 1992; 268:2069-2073.

Staali L, Morgelin M, Bjorck L, Tapper H. Steptococcus pyogenes expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. Cell Microbiol 2003 Apr; 5(4): 253-265.

Steere AC, Baxter-Lowe LA. Association of chronic, treatment resistant Lyme arthritis with rheumatoid arthritis alleles. Arthrit Rheum 1998;41: S81

Steere AC. Lyme disease. N Engl J Med 2001; 345: 115-125.

Steere AC, Falk B, Drouin EE, Baxter-Lowe LA, Hammer J, Nepom GT. Binding of outer surface protein A and human lymphocyte function-associated antigen 1 peptides to HLA-DR molecules associated with antibiotic treatment-resistant Lyme arthritis. Arthritis Rheum 2003 Feb; 48(2): 534-540.

Stevens DL, Kaplan EL eds. Streptococcal infections: clinical aspects, microbiology and molecular pathogenesis. New York, Oxford university press 2000.

Stillerman M, Bernstein SH. Streptococcal pharyngitis: Evaluation of clinical syndromes in diagnosis. Am J Dis Child 1961; 101: 476-489.

Stollerman GH. Rheumatic fever in the 21st century. Clin Infect Dis. 2001 Sep; 33(6): 806-814.

Sutton I. Paraneoplastic neurological syndromes. Curr Opinion Neurol. 2002; 15(6): 685-90.

Sutton I, Winer JB. The immunopathogenesis of paraneoplastic neurological syndromes. Clin Sci (London). 2002 May; 102(5): 475-86.

Swedo SE, Rapoport JL, Cheslow DL, Leonard HL, Ayoub EM, Hosier DM, Wald ER. High prevalence of obsessive-compulsive symptoms with repeated episodes of Sydenham's Chorea. Am J Psych 1989 Feb; 146(2): 246-249.

Swedo SE, Leonard HL, Schapiro MB, Casey BJ, Mannheim GB, Lenane MC, Rettew DC. Sydenham's chorea: Physical and psychological symptoms of St. Vitus dance. Pediatrics 1993 Apr; 91(4):706-713.

Swedo SE. Sydenham's chorea. A model for childhood autoimmune neuropsychiatric disorders. JAMA. 1994 Dec14; 272(22):1788-1791.

Swedo SE, Leonard HL, Mittleman BB, Allen AJ, Rapoport JL, Dow SP, Kanter ME, Chapman F, Zabriskie J. Identification of children with PANDAS by a marker associated with rheumatic fever. Am J Psych 1997; 154:110-112.

Swedo SE, Leonard HL, Garvey M, Mittleman B, Allen AJ, Pelmutter S, Lougee L, Dow S, Zamkoff J, Dubbert BK. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical descriptions of the first 50 cases. Am J Psych 1998 Apr; 155(2): 264-271.

Sydenham T. The entire works of Thomas Sydenham. Vol 1 & 2. London, England: Sydenham society; 1848. Reprinted by: Birmingham, Ala classics in Medicine Society: 1979.

Tanaka D, Gyobu Y, Kodama H, Isobe J, Hosorogi S, Hiramoto Y, Karasawa T, Nakamura S. emm Typing of group A streptococcus clinical isolates: identification of domnant types for throat and skin isolates. Micribiol Immunol 2002; 46(7): 419-423.

Tanaka K, Tanaka M, Igarashi S, Onodera O, Miyatake T, Tsuji S. Trial to establish an animal model of paraneoplastic cerebellar degeneration with anti-Yo antibody. 2. Passive transfer of murine mononuclear cells activated with recombinant Yo protein to paraneoplastic cerebellar degeneration lymphocytes in severe combined immunodeficiency mice. Clin Neurol Neurosurg 1995 Feb; 97(1): 101-105.

Tanaka K, Ding X, Tanaka M. Effects of antineuronal antibodies with paraneoplastic neurological syndrome on primary-cultured neurones. J Neurol Sci 2004 Jan; 217(1): 25-30.

Tanaka M, Tanaka K, Onodera O, Tsuji S. Trial to establish an animal model of paraneoplastic cerebellar degeneration with anti-Yo antibody. 1. Mouse strains bearing different MHC molecules produce antibodies on immunization with recombinant Yo protein, but do not cause Purkinje cell loss. Clin Neurol Neurosurg. 1995 Feb; 97(1): 95-100.

Tanaka M, Tanaka K, Tsuji S, Kawata A, Kojima S, Kurokawa T, Kira J, Takiguchi M. Cytotoxic T cell activity against the peptide, AYRARALEL, from Yo proteins of patients with the HLA A24 or B27 supertype and paraneoplastic cerebellar degeneration. J Neurol Sci. 2001; 15, 88 (1-2): 61-5.

Tanaka M, Maruyama Y, Sugie M, Motizuki H, Kamakura K, Tanaka K. Cytotoxic T cell activity against peptides of Hu protein in the anti-Hu syndrome. J Neurol Sci. 2002 Sep 15; 201(1-2): 9-12.

Taneja V, Mehra NK, Reddy KS, Narula J, Tandon R, Vaidya MC, Bhatia ML: HLA-DR/DQ antigens and reactivity to B cell alloantigen D8/17 in Indian patients with rheumatic heart disease. Circulation 1989 Aug; 80(2): 335-340.

Taranta A, Stollerman GH. The relationship of Sydenham's chorea to infection with Group A Streptoccci. Am J Med 1956; 20:170.

Taylor JR, Morshed SA, Parveen S, Mercadante MT, Scahill L, Peterson BS, King RA, Leckman JF, Lombroso PJ. An animal model of Tourette's syndrome. Am J Psych 2002 Apr;159(4): 657-660.

Teixeira AL, Cardoso F, Maia DP, Cunningham MC. Sydenham's chorea may be a risk factor for drug induced parkinsonism. J Neurol Neurosurg Psych 2003 Sep; 74(9): 1350-1351.

Terreri MT, Roja SC, Len CA, Faustino PC, Roberto AM, Hilário MO. Sydenham's chorea-clinical and evolutive characteristics. Sáo Paulo Med J 2002 Jan 3; 120(1): 16-19.

Thayer WS. An analysis of 808 cases of chorea with special reference to cardiovascular manifestations. JAMA 1906; 47: 1352-1354.

Theofilopoulos AN, Koundouris S, Kono DH, Lawson BR. The role of INF-gamma in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity. Arthr Res 2001; 3(3): 136-141.

The Tourette syndrome association International consortium for genetics: A complete genome screen in sib pairs affected by Gilles de la Tourette syndrome. Am J Hum Genet 1999; 65: 1428-1436.

Thern A, Stenberg L, Dahlbäck B, Lindahl G. Ig-binding surface proteins of streptococcus pyogenes also bind human C3b-binding protein (C3BP), a regulatory component of the complement system. J Immunol 1995 Jan 1;154(1): 375-386.

Tiesler E, Hepp M. Comparison of value of four immunological methods (AST, ADNase B, AHy, ANADase) by A-streptococcal infections and their significance in the routine diagnostic. Immun Infekt 1976;4(4): 190-5. German

Tilelli CQ, Martins AR, Larson RE, Garcia-Cairasco N. Immunohistochemical localisation of myosin Va in the adult rat brain. Neurosci 2003; 121 (3): 573-586.

Todd E. Antigenic streptococcal hemolysis. J Exp Med 1932;55: 267-280.

Traill Z, Pike M, Byrne J. Sydenham's chorea: a case showing reversible striatal abnormalities on CT and MRI. Dev Med Child Neurol 1995 Mar; 37(3): 270-273.

Treser G, Seman M, McVicar M, Franklin M, Ty A, Sagel J, Lange K. Antigenic streptococcal components in acute glomerulonephritis Science 1969 Feb 14; 163(868): 676-677.

Trifiletti RR, Packard AM. Immune mechanisms in pediatric neuropsychiatric disorders. Tourette's syndrome. OCD, and PANDAS. Child Adolesc Psych Clin N Am. 1999 Oct; 8(4): 767-775.

Trifiletti RR, Bandele AN: Serum antibodies to specific brain proteins in patients with tics, Tourette's syndrome, or obsessive-compulsive disorder. Ann Neurol 2000; 48(3): 511-2.

Trifiletti RR, Bandele AN. Antibodies to the calpain-calpastatin complex in patients with tics, Tourette syndrome, or obsessive-compulsive disorder. Ann Neurol 2000; 48: 542 (abstract).

Tsai PJ, Lin YS, Kuo CF, Lei HY, Wu JJ. Group A streptococcus induces apoptosis in human epithelial cells. Infect Immun 1999 Sep; 67(9): 4334-4339.

Tsang RS. The relationship of Campylobacter jejuni infection and the development of Guillain-Barre syndrome. Curr Opin Infect Dis 2002 Jun; 15 (3): 221-228.

Tsunoda I, Kuang LQ, Fujinami RS. Induction of autoreactive CD8+ cytotoxic T cells during Theiler's murine encephalomyelitis virus infection: implications for autoimmunity. J Virol 2002 Dec; 76(24): 1283-1244.

Tullio AN, Bridgman PC, Tresser NJ, Chan CC, Conti MA, Adelstein RS, Hara Y. Structural abnormalities develop in the brain after ablation of the gene encoding nonmuscle myosin II-B heavy chain. J. Comp Neurol 2001 Apr; 433(1): 62-74.

Turner JC, Hayden GF, Kiselica D, Lohr J, Fishburne CF, Murren D. Association of group C beta-hemolytic streptococci with endemic pharyngitis among college students. JAMA 1990 Nov 28; 264(20): 2644-2647.

Ueta H, Nagasawa H, Oyabu-Manabe Y, Toida K, Ishimura K, Hori H. Localisation of enolase in synaptic plasma membrane as an alphagamma heterodimer in rat brain. Neurosci Res 2004; 48 (4): 379-386.

Von Santha K. Ueber Gefassveranderungen im Zentralnervensystem bei Chorea Rheumatica. Arch Path Anat. 1932; 287: 405.

van Dijk JG, van der Velde EA, Roos RA, Bruyn GW. Juvenile Huntington disease. Hu Genetic 1986 Jul; 73(3): 235-239.

van de Beek D, de Gans J, Spanjaard L, Sela S, Vermeulen M, Dankert J. Group a streptococcal meningitis in adults: report of 41 cases and a review of the literature. Clin Infect Dis 2002 May1; 34(9): e32-36.

Veasy LG, Tani LY, Hill HR. Persistence of acute rheumatic fever in the intermountain area of the United States. J Pediatr 1994 Jan; 124(1): 9-16.

Verbeek MM, De Jong D, Kremer HP. Brain-specific proteins in cerebrospinal fluid for the diagnosis of neurodegerative diseases.Ann Clin Bochem 2003 Jan; 40 (Pt1): 25-40.

a. Vincent A, Lily O, Palace J. Pathogenic autoantibodies to neuronal proteins in neurological disorders. J Neuroimmunol 1999 Dec;100(1-2): 169-180.

b. Vincent A. Immunology of the neuromuscular junction and presynaptic nerve terminal. Curr Opin Neurol 1999 Oct; 12(5): 545-551.

Walker RW, Keir G, Johnson MH, Thompson EJ. A rapid method for detecting oligoclonal IgG in unconcentrated CSF, by agarose isoelectric focusing, transfer to cellulose nitrate and immunoperoxidase staining. J Neuroimmunol 1983 Apr. 4(2);141-148.

Walkup JT, LaBuda MC, Singer HS, Brown J, Riddle MA, Hurko O: Family study and segregation analysis of Tourette syndrome: evidence for a mixed model of inheritance. Am J Hum Genet 1996 Sep; 59(3): 684-693.

Wallace MR, Garst PD, Papadimos TJ, Oldfield EC 3rd. The return of acute rheumatic fever in young adults. JAMA 1989 Nov 10; 262(18): 2557-2561.

Wannamaker LW. The differentiation of three distinct desoxyribonucleases of group A streptococci. J Exp Med 1958 Jun 1; 107(6): 797-812.

Wannamaker LW. The paradox of the antibody response to streptodornase: the usefulness of antidesoxyribonuclease B as an indication of of streptococcal infection in patients with acute rheumatic fever. Am J Med 1959 Oct; 27: 567-574.

Wannamaker LW. Diagnosis of pharyngitis: clinical and epidemiologic features. Pharyngitis: Management in an era of declining Rheumatic fever. New York: Praeger 1984; 33-46.

Waston DW. Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. J Exp Med 1960; 111:255-283.

Watanabe-Ohnishi R, Aelion J, LeGros L, Tomai MA, Sokurenko EV, Newton D, Takahara J, Irino S, Rashed S, Kotb M. Characterization of unique human TCR V beta specificities for a family of streptococcal superantigens represented by rheumatogenic serotypes of M protein. J Immunol 1994 Feb 15; 152(4):2066-2073.

Weber F, Rudel R, Aulkemeyer P, Brinkmeier H. Anti-GM1 antibodies can block neuronal voltage-gated sodium channels. Muscle Nerve 200 Sep; 23(9): 1414-20.

Weetman AP. Determinants of autoimmune thyroid disease. Nat Immunol 2001 Sep; 2(9):769-770.

Weindl A, Kuwert T, Leenders KL, Poremba M, Grafin von Einsiedel H, Antonini A, Herzog H, Scholz D, Feinendegen LE, Conrad B. Increased striatal glucose consumption in Sydenham's chorea. Mov Dis 1993 Oct;8(4): 437-444.

Wendlandt JT, Grus FH, Hansen BH, Singer HS: Striatal antibodies in children with Tourette's syndrome: multivariate discriminant analysis of IgG repertoires. J.Neuroimmunol. 2001 Sep 3; 119(1):106-113

Wessels MR, Moses AE, Goldberg JB, DiCesare TJ. Hyaluronic acid capsule is a virulence factor mucoid group A streptococci. Proc Natl Acad Sci USA 1991 Oct 1;88 (19): 8317-8321.

White AV, Hoy WE, McCredie DA. Childhood post-streptococcal glomerulonephritis as a risk factor for chronic renal disease in later life. M J Aus 2001 May 21; 174(10): 492-496.

Whitney KD, McNamara JO. Autoimmunity and neurological disease: antibody modulation of synaptic transmission. Annu Rev Neurosci 1999; 22: 175-195.

Wilson MG, Schweitzer M. Pattern of hereditary susceptibility in rheumatic fever. Circulation 1954 Nov;10(5): 699-704.

Wilson SAK. Progressive lenticular degeneration: a familial nervous disease associated with cirrhosis of the liver. Brain 1912; 34: 295-509.

Witebsky E, Rose NR, Terplan K, Paine JR, Egan RV. Chronic thyroiditis and autoimmunization. JAMA 1957 Jul; 164(13): 1439-1447.

Wyckoff M, Rodbard D, Chrambach A. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate-containing buffers using multiphasic buffer systems:properties of the stack, valid Rf-measurement, and optimised procedure. Anal Biochem 1977 Apr; 78(2): 459-482.

Yamano Y, Nagai M, Brennan M, Mora CA, Soldan SS, Tomaru U, Takenouchi N, Izumo S, Osame M, Jacobson S. Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA and proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1 associated myelopathy (HAM/TSP). Blood 2002 Jan 1; 99(1): 88-94.

Yamashiro K, Tomiyama N, Ishida A, Terada Y, Mukawa J, Yoshii Y, Tasker RR, Albe-Fessard D. Characteristics of neurones with high frequency discharge in the central nervous system and their relationship to chronic pain. Experimental and clinical investigations. Stereotact Funct Neurosurg 1997; 68(1-4 Pt1): 149-154.

Yang LC, Soprey PR, Wittner MK, Fox EN. Streptococcal induced cell mediated immune destruction of cardiac myofibers in vitro.1977 Aug 1; 146: 344-360.

Yarwood JM, Leung DY, Schlievert PM. Evidence for the involvement of bacterial superantigens in psoriasis, atopic dermatitis, and Kawasaki syndrome. FEMS Microbiol Lett 2000 Nov 1;192(1):1-7

Yoshioka T, Matsutani T, Toyosaki-Maeda T, Suzuki H, Uemura S, Suzuki R, Koike M, Hinuma Y. Relation of streptococcal pyrogenic exotoxin C as a causative superantigen for Kawasaki disease. Pediatr Res 2003 Mar; 53(3): 403-410.

Zabriskie JB. Mimetic relationship between group A streptococci and mammalian tissues. Adv Immunol 1966; 7: 147-188.

Zabriskie JB, Lavenchy D, Williams RC jr, Fu SM, Yeadon CA, Fotino M, Braun DG. Rheumatic fever-associated B cell alloantigens as identified by monoclonal antibodies. Arthritis Rheum 1985 Sep; 28(9): 1047-1051.

Zabriskie JB, Hsu KC, Seegal BC. Heart-reactive antibody associated with rheumatic fever: Characterisation and diagnostic significance. Clin Exp Immunol 1970 Aug; 7(2): 147-159.

Zeman A, McLean B, Keir G, Luxton R, Sharief M, Thompson E. The significance of serum oligoclonal bands in neurological diseases. J Neurol Neurosurg Psych 1993 Jan; 56 (1): 32-35.

Ziegler LH. The neuropathology findings in case of Sydenham's chorea. J Nerv & Ment Dis. 1927; 65: 273.

9.11 Publications from this thesis

1. Dale RC, Candler PM, Church AJ, Wait R, Pocock JM, Giovannoni G. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. J Neuroimmunol. 2005 Dec 11

2. Dale RC, Heyman I, Giovannoni G, Church A. Incidence of anti-brain antibodies in children with obsessive-compulsive disorder. Br J Psychiatry. 2005 Oct;187:314-9

3. Martino D, Branson JA, Church AJ, Candler PM, Livrea P, Giovannoni G, Dale RC. Soluble adhesion molecules in acute disseminated encephalomyelitis.Pediatr Neurol. 2005 Oct;33(4):255-8.

4. Martino D, Branson JA, Church AJ, Candler PM, Livrea P, Giovannoni G, Dale RC. Soluble adhesion molecules in acute disseminated encephalomyelitis. Pediatr Neurol. 2005 Oct;33(4):255-8.

5. Martino D, Church AJ, Defazio G, Dale RC, Quinn NP, Robertson MM, Livrea P, Orth M, Giovannoni G. Soluble adhesion molecules in Gilles de la Tourette's syndrome.J Neurol Sci. 2005 Jul 15;234(1-2):79-85.

6. Martino D, Tanner A, Defazio G, Church AJ, Bhatia KP, Giovannoni G, Dale RC. Sydenham's chorea: historical documents from a British paediatric hospital.Arch Dis Child. 2005 May;90(5):507-11.

7. Chapman MD, Hartley JC, Furrows SJ, Ridgeway GL, Thompson EJ, Church AJ, Candler PM, Giovannoni G. Lack of serum oligoclonal antibody responses to Chlamydophila pneumoniae in multiple sclerosis. Eur Neurol. 2005;53(2):81-3. Epub 2005 Apr 5.

8. Edwards MJ, Dale RC, Church AJ, Trikouli E, Quinn NP, Lees AJ, Giovannoni G, Bhatia KP. Adult-onset tic disorder, motor stereotypies, and behavioural disturbance associated with antibasal ganglia antibodies. Mov Disord. 2004 Oct;19(10):1190-6.

9. Martino D, Church AJ, Dale RC, Giovannoni G. anti-basal ganglia antibodies and PANDAS.
Mov Disord. 2005 Jan;20(1):116-7

10. Harrison NA, Church A, Nisbet A, Rudge P, Giovannoni G. Late recurrences of Sydenham's chorea are not associated with anti-basal ganglia antibodies. J Neurol Neurosurg Psychiatry. 2004 Oct;75(10):1478-9.

11. Edwards MJ, Trikouli E, Martino D, Bozi M, Dale RC, Church AJ, Schrag A, Lees AJ, Quinn NP, Giovannoni G, Bhatia KP. Anti-basal ganglia antibodies in patients with atypical dystonia and tics: a prospective study. Neurology. 2004 Jul

13;63(1):156-8.

12. Church AJ, Dale RC, Giovannoni G. Anti-basal ganglia antibodies: a possible diagnostic utility in idiopathic movement disorders? Arch Dis Child. 2004 Jul;89(7):611-4

13. Dale RC, Heyman I, Surtees RA, Church AJ, Giovannoni G, Goodman R, Neville BG. Dyskinesias and associated psychiatric disorders following streptococcal infections. Arch Dis Child. 2004 Jul;89(7):604-10.

14. Leger GC, Johnson N, Horowitz SW, Dale RC, Church AJ, Mesulam MM. Dementia-like presentation of striatal hypermetabolic state with antistriatal antibodies responsive to steroids. Arch Neurol. 2004 May;61(5):754-7

15. Edwards MJ, Dale RC, Church AJ, Giovannoni G, Bhatia KP. Dystonic syndrome associated with anti-basal ganglia antibodies.J Neurol Neurosurg Psychiatry. 2004 Jun;75(6):914-6.

16. Miranda M, Cardoso F, Giovannoni G, Church A. contraceptive induced chorea: another condition associated with anti-basal ganglia antibodies.J Neurol Neurosurg Psychiatry. 2004 Feb;75(2):327-8. Review.

17. Dale RC, Church AJ, Heyman I. encephalitis after varicella zoster infection complicated by Tourettism. Mov Disord. 2003 Dec;18(12):1554-6.

18. Dale RC, Church AJ, Surtees RA, Lees AJ, Adcock JE, Harding B, Neville BG, Giovannoni G. Encephalitis lethargica syndrome: 20 new cases and evidence of basal ganglia autoimmunity. Brain. 2004 Jan;127(Pt 1):21-33.

19. Church AJ, Dale RC, Lees AJ, Giovannoni G, Robertson MM. Tourette's syndrome: a cross sectional study to examine the PANDAS hypothesis. J Neurol Neurosurg Psychiatry. 2003 May;74(5):602-7.

20. Church AJ, Dale RC, Cardoso F, Candler PM, Chapman MD, Allen ML, Klein NJ, Lees AJ, Giovannoni G. CSF and serum immune parameters in Sydenham's chorea: evidence of an autoimmune syndrome? J Neuroimmunol. 2003 Mar;136(1-2):149-53.

21. Dale RC, Church AJ, Surtees RA, Thompson EJ, Giovannoni G, Neville BG. Post-streptococcal autoimmune neuropsychiatric disease presenting as paroxysmal dystonic choreoathetosis. Mov Disord. 2002 Jul;17(4):817-20.

22. Dale RC, Church AJ, Benton S, Surtees RA, Lees A, Thompson EJ, Giovannoni G, Neville BG. Post-streptococcal autoimmune dystonia with isolated bilateral striatal necrosis. Dev Med Child Neurol. 2002 Jul;44(7):485-9

23. Church AJ, Cardoso F, Dale RC, Lees AJ, Thompson EJ, Giovannoni G. Anti-basal ganglia antibodies in acute and persistent Sydenham's chorea. Neurology. 2002 Jul 23;59(2):227-31.

24. Dale RC, Church AJ, Wyatt MC, Sharma S, Chong KW, Cox TC. Normal MRI neuroimaging and acute dancing eyes syndrome. Dev Med Child Neurol. 2002 May;44(5):356.

25. Hartley LM, Ng SY, Dale RC, Church AJ, Martinez A, de Sousa C. Immune mediated chorea encephalopathy syndrome in childhood.Dev Med Child Neurol. 2002 Apr;44(4):273-7

26. Church AJ, Dale RC. Antistreptolysin-O titers: implications for adult PANDAS. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections.
Am J Psychiatry. 2002 Feb;159(2):320

27. Ramachandran V, Church A, Giovannoni G, Bhatia KP. Anti-basal ganglia antibodies are absent in patients with primary blepharospasm.Neurology. 2002 Jan 8;58(1):150.

28. Dale RC, Church AJ, Cardoso F, Goddard E, Cox TC, Chong WK, Williams A, Klein NJ, Neville BG, Thompson EJ, Giovannoni G. Poststreptococcal acute disseminated encephalomyelitis with basal ganglia involvement and auto-reactive antibasal ganglia antibodies.Ann Neurol. 2001 Nov;50(5):588-95.

10 Appendices

10.1 Appendix I. Indirect Immunofluorescence

Human tissue blocks and sections were kindly provided by the Neurological diseases tissue bank, Institute of Neurology, Queen Square. London WC1N 3BG.

Rat tissue sections were purchased from The Binding Site.

Sections were mounted with fluid containing 75% glycerol (Sigma G5516) and 25% PBS (Sigma P4417) which was pipetted onto each slide and a glass cover slip was placed on top of the section ensuring air bubbles were not present.

10.2 Appendix II. Brain Homogenate preparation T-per reagent (Perbio 78510)

T-Per is a cocktail of detergents, diluted in 25mM bicine, 150mM sodium chloride with a pH of 7.6. The tissue was weighed and 1mL of T-per was added per gram of tissue. Homogenisation occurred in the presence of protease inhibitor cocktail (Sigma P-8340) using a ratio of 1 part inhibitor to 10 parts of tissue. Centrifuge the sample at 10,000 rpm for 5 mins to pellet the debris. Collect supernatant.

10.3 Appendix III Enzyme linked immunoabsorbant assay

Anti-basal ganglia ELISA template

Column 1-6 (IF positive SC) Column 7-9 (IF negative serum)

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK
B	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100
C	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200
D	1/250	1/250	1/250	1/250	1/250	1/250	1/250	1/250	1/250	1/250	1/250	1/250
E	1/300	1/300	1/300	1/300	1/300	1/300	1/300	1/300	1/300	1/300	1/300	1/300
F	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400
G	1/500	1/500	1/500	1/500	1/500	1/500	1/500	1/500	1/500	1/500	1/500	1/500
H	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000

ELISA coating buffer: Carbonate buffer: Stock solution 0.5M, 13.85g anhydrous sodium carbonate (BDH 102405Y) 26.1g sodium hydrogen carbonate (BDH 102475W) in 1 litre of distilled water. Working strength 1/10 dilution (0.05M solution)

Acetate buffer for colour reagent: 0.2M Stock solution 136g sodium acetate trihydrate (BDH 102363P) and 22.5ml acetic acid (BDH 100016X), adjust pH to 5.1 and make up to 5 litres with distilled water. Dilute 1/10 for working strength solution (0.02M).

ELISA Colour reagent: o-Phenylenediamine dihydrochloride (OPD), (Sigma P1526). 1g of OPD to 100ml of 0.2M acetate buffer and one ml aliquots were stored at -20 until use. One aliquot was added to 10ml of distilled water and 11 µl of hydrogen peroxide (Sigma H1009) added just before use.

10.4 Appendix IV Western Immunoblotting (mini-gels):

500µl Multimark, multi-coloured molecular weight standard (3-188 kDa), (Invitrogen LC5925)

LDS sample buffer; 10% glycerol, 141mM Tris base, 106nM Tris-HCL, 2% LDS, 0.51mM EDTA, 0.175mM phenol red, 0.22mM brilliant blue, dissolved in ultra pure water and adjusted to pH 8.5. (Invitrogen NP0007).

Reducing agent, 0.05M dithiothreitol (Sigma D9163)

NuPage MES (2-(N-Morpholino) ethane sulfonic acid) running buffer;
50mM MES, 50mM Tris base, 0.1% SDS, 1mM EDTA and adjusted to pH 7.3.
(Invitrogen NP0002).

NuPage MOPS (3-(N-Morpholino) propane sulfonic acid) running buffer;
50mM MOPS, 50mM T Tris base, 0.1% SDS, 1mM EDTA and adjusted to pH 7.7,
(Invitrogen NP0001)

Electro-blotting transfer buffer; 25mM Bicine, 25mM Bis-tris, 1mM EDTA, 0.05mM chlorobutanol and adjusted to pH 7.2. (Invitrogen NP0006). Working strength buffer: 100ml Methanol (Fisher Scientific M/3950/17) 425ml distilled water and 25 ml transfer buffer concentrate (Invitrogen NP0006).

NuPage 4-12% bis-tris, precast 10 well gels. (Invitrogen NP0321).

NuPage 4-12% bis-tris, precast 2D well gels. (Invitrogen NP0326)

Colloidal blue staining kit (cat: LC6025). To stain one gel, 20ml of stain A was added to 55mL distilled water containing 20ml methanol (Fisher Scientific M/3950/17). The gel was left at room temperature for 10 minutes with gentle shaking before adding 5ml of stain B.

Fixation and de-staining solution for comassie staining of gel:
35ml Ethanol (Fisher Scientific E/0600/17), 10ml of glacial acetic acid (A/0360/PB17) and 55ml distilled water.

Colourmetric substrate for Western blotting: 50mg of the substrate 4-chloro-1-naphthol (Sigma C8890) was dissolved in 20ml of ethanol (Fisher Scientific E/0600/17) and 100ml of working strength acetate buffer. 100µl of 30% w/w hydrogen peroxide (Sigma H1009) was added just before use.

Minigel drying kit (cat: N12387)
After staining the gel the gel was transferred to the gel dry solution for 10minutes with gentle rocking. Two pieces of cellophane were also thoroughly soaked for 5minutes. The gel was laid on one sheet of cellophane and the other placed on top. I found that gentle rolling with a glass rod removed excess air and prevented 'cracking'. The gel was placed

in the gel drying frame and left for two days at room temperature. I found that it was important to keep out of sunlight as uneven drying lead to 'cracking'.

10.5 Appendix V Two-dimensional electrophoresis; Minigel system

500 µl IEF coloured marker (Invitrogen 39212-01).

IEF sample buffer pH 3-10; 20mM Arginine, 20mM Lysine and 15% glycerol (Invitrogen LC5311)

IEF Anode buffer: pH 3-10; 85% phosphoric acid (Invitrogen LC5300)

IEF Cathode buffer: pH 3-10; 40mM lysine (Invitrogen LC5300)

pH 3-10 pre-cast 1mm, 10 well gels (Invitrogen EC6655A)

Gel preservation fluid: 75mL ethanol (Fisher Scientific E/0600/17), 12mL glycerol (Sigma G5516) and 160ml of distilled water

Two-dimensional electrophoresis using large gel format

Protein solubilisation buffer was a solution containing 8M Urea, (Amersham Pharmacia 17-1319-01) and 4% (w/v) CHAPS, (zwitterionic, non-denaturing detergent), (Amersham Pharmacia 17-1314-01) which had been dissolved in distilled water.

Equilibration solution: 50mM Tris-HCL (Amersham Pharmacia 17-1321-01), 6M Urea (Amersham Pharmacia 17-1321-01), 30% v/v Glycerol (Amersham Pharmacia 17-1325-01), 2% w/v SDS (Amersham Pharmacia 17-1313-01), 0.001% bromophenol blue (Amersham Pharmacia 17-1329-01) and 0.05M DTT (Amersham Pharmacia 17-1318-01).

10.6 Appendix VI Immunoprecipitation

Binding buffer/wash buffer 1: 0.14M NaCl, 0.008M Na_2PO_4, 0.002M KPO_4, 0.01M KCL dissolved in 500ml of ultra pure water and adjusted to pH 7.4. (Perbio 45225).

Quenching/wash buffer 2: 25mM Tris-HCL, 0.15M NaCl dissolved in 500ml of ultra pure water and adjusted to pH 7.2 (Perbio 45225).

Elution buffer: 50ml solution of primary amine, pH 2.8 (Perbio 45225).

10.7 Appendix VII Streptococcal and human enolase protein-protein sequence comparison

```
Query: 4    ITDVYAREVLDSRGNPTLEVEVYTESGAFGRGMVPSGASTGEHEAVELRDGDKSRYLGLG
63
             I  ++ARE+LDSRGNPT+EV++YT  G F R  VPSGASTG +EA+ELRDGDK RYLG G
Sbjct: 3    IEKIWAREILDSRGNPTVEVDLYTAKGLF-RAAVPSGASTGIYEALELRDGDKQRYLGKG
61

Query: 64   TQKAVDNVNNIIAEAII--GYDVRDQQAIDRAMIALDGTPNKGKLGANAILGVSIAVARA
121
               KAVD++N+ IA A+I  G  V +Q+ +D  M+ LDGT NK K GANAILGVS+AV +A
Sbjct: 62   VLKAVDHINSTIAPALISSGLSVVEQEKLDNLMLELDGTENKSKFGANAILGVSLAVCKA
121

Query: 122  AADYLEVPLYTY---LGGFNTKVLPTPMMNIINGGSHSDAPIAFQEFMIMPVGAPTFKEG
178
              A  E+PLY +   L G +  +LP P  N+INGGSH+   +A QEFMI+PVGA +F++
Sbjct: 122  GAAERELPLYRHIAQLAGNSDLILPVPAFNVINGGSHAGNKLAMQEFMILPVGAESFRDA
181
```

```
Query: 179 LRWGAEVFHALKKILKER--GLVTAVGDEGGFAPKFEGTEDGVETILKAIEAAGYEAGEN
236
           +R GAEV+H LK ++K++     T VGDEGGFAP      + +E + +AI+ AGY
Sbjct: 182 MRLGAEVYHTLKGVIKDKYGKDATNVGDEGGFAPNILENSEALELVKEAIDKAGYT---E
238

Query: 237 GIMIGFDCASSEFYDKERKVYDYTKFEGEGAAVRTSAEQVDYLEELVNKYPIITIEDGMD
296
            I+IG D A+SEFY   +   D+ K   + +   T  +     ++ V  YP++IED  D
Sbjct: 239 KIVIGMDVAASEFYRDGKYDLDF-KSPTDPSRYITGDQLGALYQDFVRDYPVVSIEDPFD
297

Query: 297 ENDWDGWKVLTERLGKRVQLVGDDFFVTNTEYLARGIKENAANSILIKVNQIGTLTETFE
356
           ++DW  W   T  +G  +Q+VGDD  VTN + + R ++E A N +L+KVNQIG++TE  +
Sbjct: 298 QDDWAAWSKFTANVG--IQIVGDDLTVTNPKRIERAVEEKACNCLLLKVNQIGSVTEAIQ
355

Query: 357 AIEMAKEAGYTAVVSHRSGETEDSTIADIAVATNAGQIKTGSLSRTDRIAKYNQLLRIED
416
           A ++A+E G+  +VSHRSGETED+ IAD+ V    GQIKTG+  R++R+AKYNQL+RIE+
Sbjct: 356 ACKLAQENGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLMRIEE
415

Query: 417 QLGEVAQYKG 426
           +LG+ A++ G
Sbjct: 416 ELGDEARFAG 425
```

10.8 Appendix VII Enolase activity assay

Purification of IgG: Protein A column (Amersham Pharmacia 17-042-03) primed with binding buffer (20mM sodium phosphate pH 7). Serum injected into the column using a syringe. The column was washed with 5-10 column washes (1mL volumes) and eluted IgG using 0.1M citric acid pH 3-6. Fractions assessed by Western immunoblotting and immunofixation electrophoresis for IgG.

Enzyme activity: the substrate consisted of 100mM HEPES buffer (Sigma H0887), pH 7 which contained 3.3mM magnesium sulphate (Sigma M7506), 0.2mM NADH (Sigma N5130), 0.3mM 2-phosphoglycerate (Sigma P-0257), 1.2mM ADP (Sigma A2754), 10.3 IU lactate dehydrogenase (Sigma L-3888) and 2.7 IU of pyruvate kinase (Sigma P-9136) in a final volume of 1ml.

10.9 Appendix VIII Suppliers

Amersham Pharmacia Biosciences

Little Chalfont

Buckingshire

HP7 9NA

BDH-part of

VWR International Ltd

Merck House

Poole

Dorset

BH15 1TD

Tel: 1202 660444

Fisher Scientific UK

Bishop Meadow Road

Loughborough

Leicestershire

LE11 5RG

Tel: 01509 231166

Invitrogen Ltd

Inchinnan Business Park

3 Fountain Drive

Paisley

Scotland

PA49RF

Tel: 0800 269 210

Perbio Science UK limited

Century House

Tattenhall

Cheshire

CH3 9RJ

Tel: 01829 771 744

Public Health Laboratory service

61 Colindale Avenue

Colindale

London

NW9 5HT

0208 200 4400

Sigma-Aldrich company Ltd

Fancy Road

Poole

Dorset

BH12 4QH

Tel: 0800 717181

The Binding site

P.O. BOX 4073

Birmingham

B29 6AT

Tel: 0121 414 2000

10.10 Appendix VIIII Publications

ELSEVIER

Journal of Neuroimmunology 172 (2006) 187 – 197

Journal of
Neuroimmunology

www.elsevier.com/locate/jneuroim

Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease

Russell C. Dale [a,b], Paul M. Candler [a], Andrew J. Church [a], Robin Wait [c], Jennifer M. Pocock [a], Gavin Giovannoni [a,*]

0165-5728/$ - see front matter
doi:10.1016/j.jneuroim.2005.10.014